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Two stable variants of *Burkholderia pseudomallei* strain MSHR5848 express broadly divergent *in vitro* phenotypes associated with their virulence differences

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ABSTRACT

Burkholderia pseudomallei (*Bp*) the biothreat agent of melioidosis, causes disease ranging from acute and rapidly fatal to protracted and chronic. *Bp* is highly infectious by aerosol, can cause severe disease with nonspecific symptoms, and is multiply antibiotic resistant. However, no vaccine exists. Unlike many *Bp* strains, which exhibit random variability in traits such as colony morphology, the Department of Defense's Unified Culture Collection solid medium-prepared stock of *Bp* strain MSHR5848 (designated BURK178) exhibited two distinct and relatively stable colony morphologies on sheep blood agar plates: a smooth, glossy, pale yellow colony (type 1) and a flat dry white colony (type 2). A stock prepared independently from one type 2 colony using liquid medium appeared to be phenotypically identical to the solid medium stock. Passage of these two variant colony types under standard laboratory conditions produced cultures composed >99.9% of the single corresponding type 1 or type 2 morphotype. However both types could switch to the other one at frequencies which varied with the growth condition. These MSHR5848 derivatives were extensively characterized to identify differences associated with type 1 and type 2 colony morphologies.: 1) microscopic morphology, and colony morphology differences on each of six differential agar media were observed between the type 1 and the type 2 morphotype; and only type 2 metabolized sugars in BCSA plates. 2-3) Antimicrobial susceptibilities and LPS features were characterized. 4) Biolog GEN III and phenotype microarray profiles revealed distinct metabolic and susceptibility differences between the variants. Analysis of the phenotype microarray narrowed the 1,920 substrates to a manageable subset which differentiates the two phenotypes. Furthermore, growth curves show that the type 1 variant grows more rapidly than the type 2 variant. 5) In mouse challenges, the type 2 variants exhibited 10-fold greater virulence than the type 1 variant. Finally, using a macrophage model, type 1 was phagocytosed and replicated to a greater extent and was more cytotoxic than type 2. In contrast, multiple locus sequence type analysis, restriction fragment length polymorphisms, and ribotyping demonstrated the genetic conservation of the variants; yet whole genome sequence comparisons showed differences, the importance of which is subject to further study. These distinct phenotypic and *in vivo* differences shown by phenotypic variants of a single *Bp* strain will be leveraged in future studies to identify *in vitro* markers associated with different stages in pathogenesis and for the development of more effective vaccines and therapeutics.

INTRODUCTION [All Refs are in Endnote file]

Burkholderia pseudomallei (*Bp*) causes melioidosis and is a CDC Tier 1 bacterial pathogen. *Bp* is a saprophytic, free-living organism which causes endemic infections in tropical regions such as Southeast Asia and Northern Australia. It is of widespread concern for reasons including its large environmental range (**ref** – Limmathurotsakul, D. et al. Nature Microb Lett., 2016), the challenges involved in disease diagnosis, treatment complications due to inherent and acquired antibiotic resistance, and its potential for adversarial use (**ref** – Wiersanga 2012; Dance IJAA 2014; Dance.JAC, 1991; Currie. Trans Royal 2000; Vidyalakshmi, 2008; Currie, PLoS 2010; Fritz, D. 2012; Naha, 2012; Naha, 2014). *Bp* is a potential biothreat agent because of its high aerosol infectivity and ability to cause severe disease with often nonspecific symptoms (**ref** - Wiersanga 2012; Fritz, D. 2012).

Bp can infect humans and animals by several routes of infection. Infections occur upon exposure to contaminated water, soil, or secretions, and through skin abrasions, inhalation, or ingestion. The disease is manifested by numerous and often generalized symptoms such as fever and malaise, ulcerating lesions of the skin and mucus membranes, pneumonia, granulomatous abscesses in multiple organs, and septicemia. Without effective treatment, the course of melioidosis can range from acute and rapidly fatal to a protracted and frequently chronic form (**ref** - Wiersanga 2012; Dance IJAA 2014; Welkos 2015). The chronic infections are commonly associated with immunocompromising conditions such as diabetes and can persist for years. Reoccurring illness is also observed and can potentially be due to reinfection or relapse of a persistent or latent infection. All of these forms, especially the more enduring ones, can be very challenging to diagnose and treat effectively **(ref** - Wiersanga 2012; Dance IJAA 2014; Fritz and Waag, D. 2012).

Bp strain MSHR5848 was originally isolated from the sputum of a patient with suspected inhalational melioidosis. A stock of MSHR5848 maintained at USAMRIID and designated BURK178, was observed to produce colony variants on solid medium. Strains of *Bp* typically exhibit variations in colony morphology and these variants often occur randomly and are not stably reproduced on subculture. However, BURK178 produced two distinct colony variants, type 1 and type 2, with numerous phenotypic differences, ranging from *in vitro* colony and metabolic characteristics to mouse virulence. Although the colony morphotypes were relatively stable under typical laboratory conditions, both type 1 and type 2 could switch to the other type at frequencies which varied with the growth condition switching, and it could potentially reflect adaptive changes that enhance fitness in a particular environment (**ref** – Vial, L. 2010; van der Woude et al. 2004; Wisniewski-Dye, F.and Vial, L. 2008; Chantratita et al., 2007; Tandhavanant et al., 2010; Gierok, 2016;). The frequent production of colony morphological variants from a single strain is a well-established phenomenon which has been described for many bacteria to include both gram-negative and –positive pathogens (**ref**- Stanton 1924; Nicholls 1930; Rogul 1972; White 2003; M. van der Woude, 2004; Chantratita 2007; Bernier et al., 2008; K. Deitsch 2009; Wisiewski-Dye and Vial_2008. Antonie van Leeuq. 94:493; Vial et al. 2010; Tandhavanant 2010; Velapatino et al., 2012; Austin, 2015; Wikraiphat, et al., 2015; Manso, A. 2014; Gierok, 2016). The morphotypic variation may be due to various mechanisms to include phase variation (reversible switch between an on/off expressing phase) or to antigenic variation (expression of various alternate forms of an antigen on the bacterial surface). Either form of colony morphotype expression can result from genetic or epigenetic events, ie., mechanisms which change the DNA sequence of a gene or affect its expression (transcriptional or translational or post-translational) without altering its sequence, respectively.

Early studies demonstrated the production by *Bp* isolates from clinical and environmental samples of two or more colony variants (**ref** – Stanton 1924; Nicholls 1930; Rogul 1972). In 1924, Stanton et al. first reported the presence of two colony morphotypes, a rough and mucoid form, isolated from a patient infected with *Bp* (previously *Bacillus whitmori*) [**ref** – Stanton 1924]. Nicholls and Cantab [**ref** –1930] isolated and characterized similar variants of *B. whitmori* from abscess material from an infected cow. The morphotypes were reversible, with the rough being more abundant than the mucoid type. The colony types were associated with *in vitro* phenotypic differences such as production of alkaline conditions in broth and potentially with alterations in *in vivo* virulence. Later, Rogul et al **(ref-**

1972) identified rough and smooth colony variants in numerous *Bp* strains from SE Asia isolated from animal and environmental sources and showed that the colony variants differed in several *in vitro* biochemical and physiological traits. Numerous more recent studies have supported the hypothesis that different colony morphotypes potentially reflect adaptive changes that enhance fitness in a particular environment (**ref** - Chantratita 2007; Tandhavanant 2010; Velapatino et al., 2012; Austin, 2015; Wikraiphat, et al., 2015; Gierok 2016).

Studies performed during the last 30 years by Chantratita and colleagues confirmed the predominance of the rough dry morphology in *Bp* strains cultured from melioidosis patients (**ref** – Chantratita et al., 2007). This morphotype was seen on Ashdown's selective agar medium in >75% to 93% of clinical isolates from hundreds of cases. However colony morphology varied greatly within and between samples and seven distinct colony morphologies of *Bp* were identified (**ref –** Chantratita et al., 2007). The rough dry morphotype I variant was present in nearly all clinical samples yielding mixed morphotypes, and it was probably the parental type from which the others arose *in vivo*. Multiple *Bp* colony morphologies were often recovered from different sites in individual patients. *In vitro*, morphotype I could switch to the other six types in apparent response to stresses such as iron limitation (**ref** –Chantratita et al., 2007); and morphotypes I, II, and III were associated with different abilities to survive and persist in cell culture and in mice. Comparable differences in sensitivity to killing by peroxide and antimicrobial peptide (AMP) were als reported (**ref** –Chantratita et al., 2007;Tandhavanant et al., 2010). Similar type switching was described by Velapinato et al. for variants recovered from a patient during the acute and relapse stages of *Bp* infection (**ref** –Velapatino et al., 2012). The morphotypes associated with adaptive changes differed greatly in protein expression compared to the parent type, as described in these studies (**ref** –Chantratita et al., 2012; Velapinato et al).

In addition to *Bp*, members of the *Burkholderia cepacia* complex (BCC) and the related species *Pseudomonas aeruginosa* (PA), exhibit colony morphology switching in association with their ability to colonize the lungs and cause severe infection in patients with cystic fibrosis (CF). CF results in production of thick mucus containing large amount of nutrients which promotes colonization and growth of many opportunistic pathogens. For example, BCC strain *B. ambifaria* produces variants which differ in colony morphology, biofilm formation, plant root colonization, and virulence in a manner suggesting that *B. ambifaria* adapts to the very different environments of CF patient lungs and the rhizosphere by reversible phase variation (**ref** – Vial et al. 2010; Bernier et al., 2008).

Such bacterial-host models may provide insights useful in the analysis of *Bp* strain MSHR5848 phenotypic variants. The pathogenesis of chronic lung infection previously hypothesized for pathogens such as PA and BCC links the increased nutrient availability within the CF airway with the selection for less virulent bacteria better adapted to long-term infections (**ref**- Rohmer Trends Micro 2011; D'Argenio, D.A. 2007.MolMic 654:512; Ferenci, T. 2005. MolMic 57:1-8). This reductive evolution involves loss of unnecessary metabolic pathways, saving energy to support persistent infection. Similarly, a unique case of melioidosis involving infection with chronic lung carriage led to reductive evolution in lung isolates of *Bp*, with losses of genes involved in secondary metabolism, environmental survival, and pathogenesis such as the *Bp* LPS and capsule (ref – Price 2013). The loss of the latter factors may have reduced the host immune response and promoted conversion of the agent to an attenuated yet persistent form. However other model systems have proposed that evolving pathogen-host interactions produce a more diverse population of phenotypically heterogeneous variants. For instance, while isolates from chronic CF infections have demonstrated a loss of LPS, other work showed that this virulence factor can be restored in other later isolates (**ref** – Cullen 2015; Lieberman, T.D. 2011. Nat Genet. 43:1275; Traverse, C. 2013; Bernier, S. 2005. J.Bact; Hancock, R.E. IAI. 42-170.1983; Madeira, A.Proteomics, 11:1313, 2011). Thus chronic infection may produce a more persistent but also more pathogenic form. The diseased lung environment appears to turn on expression of nutritional determinants and certain virulence factors in the infecting bacteria (eg., siderophores, secreted toxins, biofilm formation), which drives evolution of genetic diversity and promotes more aggressive infection (**Ref** – Palmer, Conway chap. 9; Cullen 2015; Caballero mBio 2015; Yang 2011; Bernier (2014 and refs 7-9: Foweraker, J. 2005; Mowat, E. 2011; Workentine, M. 2013; Senior, N.J. Poster 92_J. Cystic Fibrosis, vol 14, Supplement 1, S1-S156

Abstracts of 38th European Cystic Fibrosis Conference 2015; Lieberman, T. 2014. Nat. Genet. 43:82-87). Thus it appears that for microbial pathogens to persistently colonize a host they must adapt to the host and evolve with it in a way which promotes both increasing proliferation of the pathogen as well as its avoidance of host immune detection (**ref** –Cullen 2015; D'Argenio 2007; Price 2013; Bernier 2014).

Specific phenotypic and RNA expression profiles have been associated with host-associated adaptive responses of *Bp* (**refs.** –Chantratiti et al., 2007; Trandhavant et al., 2010; Chantratiti et al., 2012; Chieng et al., 2012; Velapatino, B. 2012, Price 2013; Hayden 2012). These associations were based on responses of strains in *in vitro* macrophage models of infection and on characteristics of isolates from patients with relapsing infections. There were some common themes relating to changes in gene expression associated with persistence of infection. While certain genes related to nutrient uptake and catabolism, and some virulence-related biosynthetic genes, were often down-regulated, as described previously, others were upregulated (**ref**s– Chieng 2012; Price 2013; Cullen 2015; D'Argenio 2007; Bernier 2014; Lieberman, T.D. 2011. Nat Genet. 43:1275; Traverse, C. 2013; Bernier, S. 2005. J.Bact; Hancock, R.E. IAI. 42-170.1983; Madeira, A.Proteomics, 11:1313, 2011). The latter included genes involved in anaerobic metabolic functions and energy production (ref – Chieng 2012; Velapatino 2012; Chantatiti et al., 2012;); certain responses promoting resistance to stress conditions such as reactive oxygen or nitrogen intermediates, antimicrobials, and low pH (ref - Chantatiti et al., 2007; Chantatiti et al., 2012; Velapatino 2012; Hayden 2012); and those encoding certain virulence-associated genes with major roles in intracellular growth in the host cell and cell-to-cell spread (eg., those encoding type six secretion system functions (T6SS-1) and *bimA*). Overall, the changes in gene expression and substrate utilization likely contribute to the increasing bacterial adaptive fitness and immune evasion mechanisms required for long term chronic or progressive infection (**ref** – Price 2013; Chantratiti et al., 2012; Velapatino 2012; Hayden 2012).

Findings from these and other models of infection could be leveraged to develop specific metabolism-based therapeutics which mitigate the role of "nutritional virulence" in pathogenesis (**ref** – Conway and Cohen 2015; Smith, M.D. 1997; Welkos et al 2015 mouse models; Atkins T. 2002; Chin C.Y. 2012; Bozue, J. 2011). The objectives of this study were to characterize the major variants of MSHR5848 phenotypically and genetically, and determine the mechanism of variant expression and its role in disease pathogenesis. It is hypothesized that an analysis of the phenotypic variation exhibited by *Bp* will help target *in vitro* markers associated with different infection stages and contribute to development of optimal vaccine and therapeutics for melioidosis.

Materials and Methods

Media and chemicals

Nonselective media used included sheep blood agar (SBA) plates and glycerol tryptone agar (GTA), brain heart infusion (BHI) agar and Luria broth (LB) agar. The four differential/selective media used included: OFPBL agar plates (oxidation-fermentation base-polymyxin B-bacitracin-lactose); PC/BCA (*Pseudomonas*/*Burkholderia cepacia* agar) plates with polymyxin B, ticarcillin, and dye to detect alkaline pyruvate metabolism; BSCA plates (*Burkholderia cepacia* selective agar), with polymyxin B, gentamicin, vancomycin, sucrose and lactose with dye to detect acid production (for *Bp*); and Ashdown's agar plates (AA) containing dyes and gentamicin (for *Bp*) or no antibiotic (for *Bm*). All were available commercially (Thermo Fisher-Remel) except GTA, LB agar, and AA plates which were manually prepared as directed by the manufacturer or as described previously (ref - Ashdown, 1979; Fritz et al., 2000). Liquid growth media were LB broth, glycerol tryptone broth (GTB) (ref - Fritz et al., 2000), or cation-adjusted Mueller-Hinton II Broth (MHB) (BBL™, BD Diagnostics Franklin Lake, NJ). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), and antimicrobial peptides were acquired from the following sources: Sigma/Fluka, Bachem (Torrance, CA), Biopeptek (Malvern, PA), Synthetic Biomolecules (San Diego, CA), and Peptides International (Louisville, KY).

Bacterial strains, stock preparations and characterization.

Burkholderia pseudomallei (*Bp*) strain MSHR5848 was originally isolated from human sputum in a suspected inhalational case at the Royal Darwin Hospital in Australia in 2011 and was subsequently sent to the Menzies School of Health Research (MSHR) in 2012. The strain was received by the USAMRIID Department of Defense Unified Culture Collection (UCC) in 2013 and designated BURK178.

Stocks of BURK178 were prepared using two independent methods, as described in **Fig. 1**. The first method (Solid media) was designed to capture a representative sample of the source culture and entailed subculturing the source vial on 5% Sheep blood agar (SBA) (Remel, Lenexa, KY) and allowing the culture to incubate for approximately 24 h. The colonies were harvested and suspended in TSB + 12.5% glycerol and subsequently aliquoted into single use cryovials. The second method (Liquid media) was designed to create a clonal, mid-log stock. The source vial was subcultured on 5% SBA from which a single colony was selected and inoculated into an MHB broth culture. Based on previously collected kinetic data, the culture was harvested at 9.5 h. An equal volume of MHB + 25% glycerol was added to the culture and subsequently aliquoted to single use cryovials. Individual colony morphologies of each stock were initially assessed post-production on 5% SBA and Ashdown's agar, and cellular morphologies were assessed by performing Gram stains on each observed variant. Variants were also stained with the fluorescent DNA binding dye propidium iodide (Sigma-Aldrich, St. Louis, MO). Type 1 or type 2 colonies were suspended in PBS and the suspensions were dried on microscope slides and stained with the fluorescent DNA binding dye propidium iodide. The slides were viewed on an Olympus BX51 microscope with phase contrast (100x, oil immersion objective) and fluorescence (exc 535 nm/ em 617 nm) microscopy.

Presence of colony variants in strain stocks.

Purity and colony morphology assessments were performed on the original source vial as well as the seed and production stocks of both the solid media and liquid media preparations. A 10-fold serial dilution of each stock was prepared using Dulbecco's Phosphate Buffered Saline (DPBS) as the diluent. In order to obtain single colonies in quantities between 30 and 300 per plate, 100 µl of the 10^{-6} and 10^{-7} dilutions of the solid media preparations were used. The original stock and liquid media preparations were slightly less concentrated so 50 µl the 10^{-5} and 100 µl of the10^{-6} dilutions were used instead. Both 5% SBA and Ashdown's agar were inoculated with the respective dilutions using a cell spreader and an inoculating turntable. In all cases, each dilution was plated in triplicate. The plates were incubated at 35°C in

ambient atmosphere for a total of 96 h, with observations occurring approximately every 24 h. In addition to standard colony morphology assessments, each plate was also photographed and the colonies counted during each observation period.

Each observed variant was characterized by multi-locus sequence typing (MLST), whole genome sequencing (WGS), Ribotyping, Sherlock Microbial Identification System (MIS), Vitek 2, the Biolog GEN III MicroPlate™ system, and Biolog Phenotype Microarray (see phenotypic and molecular methods below). Additionally, to characterize morphological stability, of each variant, a subsequent dilution series of each variant was prepared using a single representative colony as the starting material. The dilution series was prepared by suspending the single colony in 1ml of DPBS and doing another 10-fold serial dilution. One hundred microliters of the 10^{-6} and 10^{-7} dilutions were used to inoculate 5% SBA plates in triplicate. Plates were incubated at 35°C in ambient atmosphere for a total of 96 h, with observations occurring approximately every 24 h. In addition to standard colony morphology assessments, each plate was also photographed and the colonies counted during each observation period.

In vitro growth and variant switching frequency.

Growth in broth culture of MSHR5848 Solid Type 1 and Type 2.

Growth rates of each variant were assessed using the BioScreen C automated growth curve analysis system by Growth Curves USA. Each variant was first propagated on 5% SBA at 35°C for 48 h in ambient atmosphere. A single representative colony from each culture was then chosen to inoculate a MHB starter culture which was subsequently distributed to the wells of a BioScreen C plate. The starter culture was incubated on the BioScreen C at 37°C with continuous shaking and data were collected every 15 m at 600nm. When the starter culture reached an OD_{600} of 0.1-0.3, baseline subtracted, the starter culture was removed from the instrument and transitioned to a primary culture. The following formula was used to determine the volume of starter culture (V_s) with an OD_{600} between 0.1 and 0.3 (OD_s) that was required to add to fresh media with a volume V_m: $V_s=(0.001/OD_s)*V_m$. The new suspension was vortexed and distributed to the wells of a fresh BioScreen C plate. The primary culture was incubated for 48 h in the same manner as the starter culture.

Reversion frequency experiments.

Multiple stocks ("clones") were prepared of individual type 1 and type 2 colonies, 21 from MSHR5848 Solid, and 17 from the MSHR5848 Solid Type 2 stock. These clones were tested for reversion to the other type in the 14 growth conditions which are described in Table 3. The conditions tested in condition #1-10 corresponded to those detailed by Wikraiphat et al. (ref - IAI, 2015), #11 was described by Austin et al. (ref - mBio 2015), #12 and #14 were developed in this study and are described in Table 3, and #13 was performed according to the conditions depicted d by Butt et al. (ref -2014). Six of the type 1 clones tested had been fresh revertants of type 2 clone #1 that had been incubated in condition 9 (7 days at 37C).

Phenotyping methods and assays

All cultures used in phenotypic tests except as indicated were propagated on 5% SBA and incubated for 48 h in ambient atmosphere at the indicated temperatures.

Sherlock MIS.

Sample extracts were prepared in accordance with the Sherlock Microbial Identification System Operating Manual (MIDI, Inc., Newark, DE). Sample extracts were then analyzed by an Agilent 6850 gas chromatography system and searched against the Sherlock bio-threat library. Peak profiles for each variant were also compared using the Sherlock Microbial Identification System software, version 6.2.

Vitek® 2 Compact.

Suspensions of the test samples were prepared by transferring several isolated colonies from the agar plate to 3.0 ml of 0.45% saline and adjusting the turbidity as necessary until a 0.5–0.63 McFarland

standard was achieved using the DensiCHEK plus meter, in accordance with the Vitek® 2 Systems Product Information (bioMerieux Inc., Hazelwood, MO). Suspensions were then used to inoculate Vitek® 2 GN cards. Inoculation of cards, incubation, and analysis were done automatically by the Vitek® 2 Compact instrument.

GEN III OmniLog®.
The GEN III MicroPlate™ system was used for species identification and abridged phenotypic profiling. These plates contain 71 carbon sources and 23 antimicrobial chemicals. Inocula for all samples were prepared with inoculating fluid IF-A using protocol A in accordance with the OmniLog Data Collection Software Identification System User Guide, version 2.1 (Biolog, Inc., Hayward, CA). The GEN III MicroPlate™ was used and data were collected every 15 min using the full data logger option for 36 h. Samples remained in the instrument beyond the completion of the original 36 h incubation period and a single data point was collected at the 48 h time point. Sample identifications were produced using a combination of the GEN III database and manufacturer assisted user database. The metabolic profiles were also evaluated by exporting the threshold values using the Biolog Retrospect software, version 2.1.1.

Biolog Phenotype Microarray™ (PM).
The PM system of Biolog consists of 20 microplates and includes a total of 1920 substrates. PM 1 – PM 20 are used to phenotype strains in their ability to use different compounds as sources of carbon, nitrogen, and phosphorus or sulfur; or in their sensitivity to stressful environmental conditions such as pH extremes or high salt concentrations; and antimicrobial chemicals such as antibiotics, detergents, oxidizing agents, and others. Sets of PM plates 1–20 for each variant were prepared in accordance with the Biolog PM procedure for *E. coli* and other Gram negative bacteria. Kinetic data were collected every 15 min at 37°C (IF only show the 37and not 33C data) for 48 h and subsequently analyzed using version 1.20.02 of the File Management/Kinetic Plot and Parametric software.

LPS profiling.
B. pseudomallei MSHR5848 variants type 1 and type 2 were streaked onto SBA plates and incubated at 37°C for 48 h. From each plate, approximately 5–6 isolated colonies with uniform morphology (i.e., all type 1 or type 2) were resuspended in GTB broth and incubated at 37°C with shaking at 200 rpm overnight. The cultures were then diluted to an OD_{620} of approximately 1.0 and heat-killed at 90°C for 90 min. After sterility was confirmed, LPS was extracted using the procedure from Yi and Hackett (ref - 2000). The purified LPS samples were separated by SDS-PAGE using 10–20% Tricine gels (Thermo Fisher Scientific, Waltham, MA), and western blots were performed using a variety of monoclonal antibodies specific for *Burkholderia* LPS. These included 11G3-1, BP7 1H7, BML 11G6, BPL 30D11, BP7 2G6, BML 18F8, BP A2, 3D11, and 9D5. Peroxidase-labeled goat anti-mouse IgG was used as the secondary antibody (KPL, Gaithersburg, MD), and the blots were developed using colorimetric detection with TMB Membrane Peroxidase Substrate (KPL). Alternatively, silver staining was conducted using a method described by Tsai and Frasch (ref - 1982).

Antimicrobial sensitivity testing .
Chemical and antimicrobial peptide sensitivity testing was performed using GEN III MicroPlates™ as described above; and microtiter tests to evaluate sensitivity to selected chemicals and antimicrobial peptides (AMPs); growth or inhibition was determined by reading absorbance (A_{630}), as described previously (ref - Welkos et al, Frontiers). The reagents and procedures for the microtiter assays were those detailed previously (ref - Welkos et al, Frontiers). Briefly, the chemicals tested included selected concentrations of NaCl, nalidixic acid, the surfactant niaproof 4, reactive oxygen species (ROS) inducer paraquat dichloride, and reactive N_2 intermediate (RNI) sodium nitrite (2mM). Eight antimicrobial peptides (AMPs) were screened: cecropin A, mastoparan 7, LL-37, magainin, melittin, BMAP-18, bactenecin, and CA-MA (Kanthawong et al., 2009;Tandhavanant et al., 2010;Fox et al., 2012;Madhongsa et al., 2013). *E. coli* strain ATCC 25922 and *Bp* K96243 were used in the assays to verify activity. After

addition of the antimicrobials to the trays, the wells were inoculated with strains adjusted to a concentration of 1 x 10^6 CFU/ml in MHB. The trays were incubated and read as described for the Biolog trays and the absorbance results were recorded as resistant (R, OD_{630} >75% positive growth control); sensitive (S, OD_{630} <50% positive control); borderline (R/S, OD_{630} > 50% and <75% positive control); or very sensitive (S+, OD_{630} ≤2x the uninoculated negative control wells containing medium alone).

Persistance phenotype.
An *in vitro* assay to detect persister-type cells was performed to distinguish cells that are tolerant to, from those that are killed by, high concentrations of antibiotic. The procedure used was described previously by Butt and coworkers (ref - 2014) who identified the HicAB system as having a role in persistence in an *in vitro* antibiotic tolerance assay. The sensitivities to ciprofloxacin of cultures of MSHR5848 Solid type 1 and type 2 were evaluated. For both, the growth of a rapidly growing log phase and of a stationary phase cultures in LB with ciprofloxacin added (391 µg/ml, 100x MIC) was compared by dilution plating at intervals during incubation at 37C for30 h, as described (ref- Butt et al., 2014).

Molecular Genetics Methods

Ribotyping.
Variants were subcultured on 5% SBA and incubated for 48 h at 35°C in ambient atmosphere. Cultures of each sample were prepared for restriction digest with *Eco*RI according to the Dupont Qualicon Riboprinter® Microbial Characterization System User's Guide (Dupont Qualicon, Wilmington, DE). Since *Bp* is not in the Dupont library, the patterns were analyzed using BioNumerics version 7.5 (Applied Maths, Austin, TX).

MLST.
DNA was extracted from single colonies for each observed variant using heat lysis. PCR amplification and sequencing of seven housekeeping gene fragments was subsequently performed using primers for the *Burkholderia pseudomallei* MLST scheme, as previously described by Godoy, et al. (**ref** – Goday. D. 2003. Clin. Microbiol. 41:2068).

Whole Genome Sequence (WGS) analysis.

For DNA isolations, the strains were first propagated in glycerol tryptone broth (**ref** – Fritz, Waag 2000) starting from a single representative colony for each variant and incubated to log phase with shaking. The bacteria were harvested and genomic DNA (gDNA) was prepared using the Qiagen DNeasy Blood and Tissue kit. Libraries were prepared from sheared, digested and sized DNAs; they were sequenced on a Pacific Biosciences (PacBio) RSII. The libraries were processed, assembled, sequenced and annotated on the PacBio RS II as described previously (refs –Eid, J. Science 2009; Ladner, J. 2015; Daligault, H. 2014; JL refs 2, 3, 4 – Chin, Krumsiek, Tatusova). The type 1 and 2 sequences were aligned to the *B. pseudomallei* MSHR5848 reference and variants called as reported (refs –JL ref 5-7).

In separate experiments done to verify that the process of growing the cultures in broth did not skew sequencing results, WGS data was collected from single colonies of the variants, and type 1 and 2 variants were extracted with the Qiagen EZ1 Biorobot using the Virus 2.0 kit. Samples were diluted to 1 ng/µL and individually sequenced on the Illumina MiSeq platform. Three separate sequencing analyses were completed to improve coverage of all regions harboring variant sequence differences and to identify any reproducible findings. In brief, libraries were prepared utilizing the Nextera XT kit according to manufacturer's instructions. Libraries were sequenced with the V2 500 cycle kit (**Jeff/April** – OR: "sequenced using a 300 cycle paired end sequencing kit." – Which is correct?]. The sequencing reads were analyzed using CLC Genomics Workbench. Briefly, reads were trimmed for quality and mapped against the MSHR 5848 chromosomal sequences (GenBank number NZ_CP008909 for chromosome 1 and NZ_CP008910 for chromosome 2). Consensus sequences from these mappings were generated using an N for reference locations not covered by sequencing reads. Trimmed reads were mapped to this

consensus sequence to generate a final sequence for the type 1 and type 2 variants. These contigs were aligned and compared to identify nucleotide variants between the two variants for each chromosome.

Macrophage Infection assays

Phagocytosis assays were performed to measure the ability of the MSHR5848 strains to infect macrophages and to induce cell damage were performed as described in detail previously (Ref- Welkos Frontier). In brief, J774.A1 mouse macrophages in a 24-well tray were infected with 10-20 CFU *Bp* and incubated 1 h to allow phagocytosis of the bacteria. Samples of lysed cells were collected for viable counts ("1h"), or the infected wells were incubated for 2h in the presence of 250-500 µg/ml kanamycin to kill unphagocytosed bacteria. Lysed samples were collected ("3h") and the plate reincubated for 5 h ("8h") at which time lysed wells were sampled for viable counts. In addition, in separate wells, the extent of cytotoxicity and of cell loss was measured by trypan blue (TB) dye uptake and/or by staining with propidium iodide (PI). Live cells exclude TB and PI and are unstained under phase (TB) or fluorescence (PI), whereas dead cells are permeable and have blue (TB) or bright red (PI) stained nuclei. Cells on coverslips were alternately stained with Diff-Quik™ histologic stain to assess macrophage condition (normal versus necrotic, apoptotic, or multinucleated appearance of cells and nuclei), extent of formation of multi-nucleated giant cells (MNGC), and the relative level of residual bacterial infection. Strain-associated differences in these macrophage phenotypes were compared quantitatively as described (ref – Welkos Frontier).

Mouse Infection

BALB/c mice were challenged by the IP route with *Bp* MSHR5848 strain Solid, or its type 2 derivative (Solid Type 2), and the mice monitored for morbidity and mortality for 60 days, as described previously (ref - Welkos et al. 2015.PLoS ONE; Welkos 2016. Micro Open, or unpubl.). The day 21 and 60 survival data of both were evaluated statistically to calculate median lethal dose (LD_{50}) and 95% credible interval estimates.

Statistics

Statistical Method for PM plate analysis.

The data generated from the 20 PM plates were evaluated statistically to compare target responses of the MSHR5848 morphotypes and to rank those substrates producing the most different responses between the type 1 and 2 variants. The area under the curve (AUC) parameter was selected, and four different statistical methods were used to analyze the data: the Biolog Phenotype Microarray™ analysis software; the opm and Pipeline packages based on R language, as described previously (ref s– Vaas, L. PLoSOne 2012; Vaas, L. Bioinformatics 2013; and Vehkala, M. PLoSOne 2015); the DuctApe suite (Giardini, M. et al. 2014 Genomics); and a method using data normalized to positive control scores and employing a two-way ANOVA model and generalized linear model in SAS® proc Genmod (Fetterer, D. unpublished data). The results of these analyses were in general agreement on the trends suggested by a comparison of the variants' responses. The analysis using the PM version 1.20.02 of the File Management/ Kinetic Plot and Parametric software are presented. For an analysis using the PM software, the type 1 variant served as the test and the type 2 variant served as the reference. Since there was a notable signal in the A1 wells of plates 1-8, the A1 zero function was employed; the AUC parameter was chosen to compare the two variants. Specifically, the difference in the AUC between the two variants was analyzed to find the substrates that elicited the greatest difference in response. In instances where the type 2 variant had a greater response than the type 1 variant, a threshold of -3,000 omnilog units (OU) was used to select the most discriminating substrates. Due to an overall increased response of the type 1 variant, 2 separate thresholds were used to discriminate the substrates for which the type 1 variant had a greater response, 25,000 OU for the metabolic pathway assays and 11,000 OU for the chemical sensitivity assays.

These thresholds generated lists of a reasonable size to use in further exploration of functional significance.

Other statistical procedures.
Differences in the viable counts obtained from infected macrophages and absorbance values determined from GEN III plate experiments were evaluated by t-test or by ANOVA and Tukey multi-comparison post-tests as needed. These statistical analyses were done with GraphPad Prism ver. 5.2. The morbidity and mortality observed for mice challenged with *Burkholderia* and monitored for 60 days was analyzed. The day 21 and 60 survival data for mice challenged with *Burkholderia* were a evaluated statistically by Bayesian probit analysis, as described (ref - Welkos et al. 2015. PLoS ONE; Welkos 2016, Micro Open, or unpubl.). Variant growth curves were analyzed by two-way ANOVA on data collected at five minute intervals of the Bioscreen C program run.

RESULTS

Morphological characterization

Colony and microscopic characterizations.

B. pseudomalleli strain MSHR5848 is a strain included on a proposed strain panel of clinical isolates which have been characterized for virulence in mouse models for *Bp* infection by various routes (ref – Welkos 2015; Soffler et al, unpub.; is not on list of van Zandt, Frontiers 2012). Unlike many *Bp* strains, which are known to exhibit random variability in traits such as colony morphology, the DOD Unified Culture Collection stock of strain MSHR5848 (designated BURK178) exhibited two distinct colony and relatively stable morphologies when cultured on various differential and selective plated media.

On 5% SBA plates, BURK178 produced a smooth pale yellow colony (type 1) and a flat rough greyish white colony (type 2). **FIGURE 1-A** displays representative type 1 and type 2 colonies on SBA. On Ashdown's agar the type 2 variant exhibited colonies resembling the "Type I" colony morphology described by Chantratita, et al. (ref), while the type 1 variant primarily yielded colonies consistent with the "Type VI" colony morphology, shown in **FIGURE 1-B**. BCSA plates are commonly used for selective isolation of pathogenic *B. cepacia* complex species and for *Bp.* In addition to antibiotics for selection, the medium contains lactose and a pH indicator to detect strains capable of lactose utilization with the production of acid. As shown in **FIGURE 1 panels C and D**, type one colonies of the MSHR5848 Solid stock were lactose-negative and produced a pink color change attributed to alkaline metabolism of peptones. In contrast, the MSHR5848 Solid type 2 variant appeared to ferment lactose and produce acid conditions on BSCA as detected by the change in color to a greenish yellow.

In addition to being visually distinct on blood agar, the variants also differed at the microscopic level. Gram stains show that the type 2 variant has the typical safety pin appearance associated with *Bp* (Shea, unpubl. observations), while that characteristic is not as obvious in the type 1 variant (**FIGURE 2-A and 2-B**). Also, type 1 but not type 2 was stained with DNA-binding dye propidium iodide (**FIGURE 3 panels A - D**), a finding which supports previous observations that certain mucoid isolates of *Bp* secrete DNA (ref – Austin et al., 2015).

Analysis of colony variants in strain stocks.

Stock cultures were prepared from the BURK178 strain vial for experimental use. As shown in **FIGURE 4**, master seed and production stocks prepared on solid media from the BURK178 strain produced both type 1 and type 2 colonies. In contrast, the stock prepared in liquid media yielded almost exclusively the type 2 variant. These differences in composition of the stock cultures may be due to the different inocula used in their preparation. Whereas the liquid media preparation was started from a single type 2 colony, the solid media preparations included both colony types in the inoculum, as shown in **FIGURE 4**.

Master seed and production stocks prepared from the BURK178 source vial on solid media produced both type 1 and type 2 variants, though their ratio varied depending on stock culture as described in **Table 1**. The percentage of type 1 colonies varied from 0.35% in the source vial to 4.7% in the master seed stock, and increased to levels nearly equal (47%) to those of type 2 in the production stock. Thus the ratio of type 1 to type 2 increased significantly with each subsequent stock preparation, as will be discussed further below.

Different colony types isolated from the master seed and production stocks were collected, diluted, and plated for single colony isolation to characterize the morphotypes they in turn produced. All colonies observed in platings of individual type 2 colonies from the BURK178 master seed or production stocks exhibited type 2 morphology (**Table S-1**). Similarly, except for the production of low numbers (0 – 2.6%) of random unstable variants (mucoid or flat smooth grey morphotypes in Table S-1), 92 – 95% of the colonies produced by type 1 were also type 1. In contrast, both of the unstable variants of type 1 yielded colonies the majority of which displayed typical type 1 morphology and not the morphology of the unstable variant (Table S-1 and data not shown); this is illustrated by the results with the "mucoid"

variant from the master seed and production stocks, which produced type 1 colonies present as 79% to 91% of the total colony count (Table S-1).

Overview of phenotypic differences

To analyze the phenotypic and genotypic properties affected by the variant switching and begin to understand its mechanism, a wide range of characteristics was compared for the two major variants. These characteristics and assays performed are listed in **Table 2** and the type 1 and 2 responses summarized. As illustrated in Table 2, a large number of different phenotypes was affected by the switching process, ranging from in vitro metabolic activity to virulence for mice.

In vitro growth and variant switching frequency

Growth Curves.

The type 1 variant multiplied at a higher rate in vitro and reached stationary phase before the type 2 variant, as illustrated by the growth curves shown in **FIGURE 5**. The type 1 and type 2 growth curves were analyzed on data collected at five minute intervals of the automated Bioscreen C run, and for all comparisons, type 1 growth was significantly greater than that of type 2 (p values from 0.0037 - 0.0001 by two-way ANOVA).

Reversion frequency.

The frequency of switching between the two major colony variants present in the MSHR5848 stocks was examined by identifying conditions under which the type 1 and type 2 variants of MSHR5848 could revert or switch to the other morphotype. Multiple single colony stocks were prepared of individual variant colonies from the MSHR5848 solid medium production stock, 21 of type 1 and 17 of type 2. As described in **Table 3**, the single colony stocks were tested for reversion in 14 different growth conditions: #1-10 were the ten conditions described by Wikraiphat (IAI, 2015); #11 and #13 conditions were tested as described by Austin (ref. -mBio 2015) and Butt (ref. -2014), respectively; and #12 and #14 conditions were for this study and were conducted as described in **Table 3**. None of the type 1 single colony stocks that were tested directly exhibited switching to the type 2 morphotype (Table 3 combo). The six type 1 clones for which switching was observed had been freshly-isolated revertants of a type 2 colony stock incubated in condition 9 (7 days at 37°C). These six type 1 isolates were tested for reversion to type 2 using conditions 9, 12, and 14. Only condition #14 induced reversion of type 1 to 2, at frequencies which varied between experiments (**Table 3**, and data not shown). The type 2 variant overall reverted more frequently and in more growth conditions than was observed for type 1, although the switching frequency was variable between experiments (**Table 3**, and data not shown). Reversion of type 2 colonies to type 1 was not observed for six conditions, occurred with low frequency in conditions #2, 3, 6, and 7 (from 0.1 to 1.6% of colonies plated), and occurred more frequently in conditions #4 and 5 (up to 5.1 and 2.3%, during growth in high pH conditions or at 42°C, respectively). For the latter, the frequency of type 2 to type 1 switching increased to 30.5% when the cultures were incubated 6 days (42°C). Reversion was detected most often with condition #9 (mean of 20.9%), but was variable between experiments in both the number of type 2 single colony strains that produced revertants and the percentage of type 1 colonies produced. Condition #9 involved incubation in TSB without shaking at 37°C for 7 days.

Species identification.

Three platforms were used for comparative species identification results for the MSHR5848 variants. In the Biolog GEN III MicroPlate™ system, the type 1 variant was correctly identified as *Bp* in two out of three runs at the 36 h time point but was mis-identified as *B. thailandensis* in all 3 runs at the 48 h time point. Conversely, the identification of the type 2 variants (from MSHR Solid and Liquid stocks) varied among 4 different species of *Burkholderia* at the 36 h time point but were identified correctly as *Bp* in all 3 runs at the 48 h time point (**Table 2** and **Table 4**). In general, the type 1 variant was more metabolically active than the type 2 variant, an observation consistent with the GEN III and Biolog PM phenotyping result (described below) .

The Sherlock MIS and Vitek® 2 systems correctly identified each variant as *Bp* with high confidence calls, according to the manufacturer's classification. However, on both platforms, the raw data showed differences between the 2 variants similar to what was noted in the Biolog experiments. In the Vitek® 2 system, seven substrates were utilized by the type 1 variant but not used by the type 2 variant from either the solid or liquid stocks (D-mannose, D-cellobiose, malonate, D-sorbitol, citrate, D-maltose, coumarate) (data not shown). No other differences were observed between the three strains. Although all MSHR5848 variants were identified correctly as *Bp* by the Sherlock MIS system, there were significant peak profile differences between the two variant types, as indicated in **Table 5**. In some cases, the type 1 variant possessed peaks that the liquid and solid stock type 2 variants did not, and in other cases, the opposite was true. In some instances, while both colony types possessed a particular peak, the percent contribution of that peak to the overall profile varied significantly. In all cases, the liquid and solid stock type 2 variants were very similar.

Antimicrobial sensitivity testing and biochemical phenotyping

Antimicrobial sensitivity testing.

The sensitivities to antimicrobial chemicals were assessed in assays with the GEN III microplate of 23 antimicrobial chemicals and in separate tests with five chemicals and eight AMPS. Inter-experimental variability in *Bp* responses of the type 1 and 2 variants was observed, as documented previously (ref – Welkos Archives Micro; Wikraiphat 2015, Austin 2015, Chantratita et al., 2007;Tandhavanant et al., 2010). The only partially consistent differences between type 1 and type 2 were in sensitivity to the toxic amino acid D-serine* and the cationic AMP magainin II (ref. – Zasloff, M. 1987. PNAS 84:5449; Zasloff 2002; Korte-Berwanger, M. et al. 2013.IAI; Maas, W.K. 1950. J. Bacteriol. 60:733-745; Grula, M.M. et al. 1963. Biochim.Biophys. Acta 74:776-778; Roesch, P.L. et al. 2003. Mol. Micro 49:55-67). Type 1 was generally more sensitive than type 2 in GENIII tests with D-serine but responses to this chemical did not significantly discriminate the variants in the phenotype microarray studies. Whereas type 1 was sensitive to borderline in 3/6 assays with magainin II, type 2 was resistant (5/6) or borderline (1/6) to the AMP (data not shown).

Phenotype microarrays.

The single 96-well GEN III microplate is an abridged version of the Biolog Phenotype Microarray™ system and contains 71 carbon sources in addition to antimicrobial chemicals. In general, the type 1 variant was more metabolically active than either of the type 2 variant. A total of 32 substrates differed between the type 1 and two type 2 strains in at least one of three tests done; the 15 substrate which consistently differed between strains in all 3 tests are shown in **Table 6**. The arbitrary respiratory units are normalized on a 0-100 scale by an algorithm of the BiOLOG program, which also produces Threshold values. These were used to establish positive and positive/negative cut-off values as described in the table, and these allow differences in growth of the bacteria in the chemicals to be discriminated. It is readily apparent that type 1 can metabolize a wider range of carbon sources than the type 2 variants.

The Phenotype Microarray™ system employs 20 microplates with a total of 1,920 substrates to phenotype strains and identify strain differences in their ability to use different compounds as sources of carbon, nitrogen, and phosphorus or sulfur; or in their sensitivity to stressful environmental conditions such as pH extremes or high salt concentrations; and antimicrobial chemicals such as antibiotics, detergents, oxidizing agents, and others. The data generated from these plates was consistent with the findings from the GEN III experiments. The type 1 variant was generally more metabolically active than the type 2 variant, and the type 2 solid and liquid stock variants were very similar. The type 1 variant produced greater responses overall across all 20 plates compared to the type 2 variant. The greatest differences were observed in metabolic pathway assays, specifically in the carbon source plates, though also in resistance to certain stress conditions, eg., low pH and toxic ions. It should be noted that although the variants differed in growth rate (**Figure 5**), the PM platform measures cellular metabolism (respiration) and not the increase in cell numbers and biomass. When a strain stops multiplying or even fails to grow, it may continue to metabolize the substrate. To further examine the association between

growth rate and respiration activity, PM plates were inoculated and incubated with type 1 and type 2 and absorbances read for comparison to the Omnilog results. The type 1 and 2 turbidity data agreed with the metabolic activity for the carbon, sulfur, and dipeptide nitrogen substrates shown in **Tables 7A and 7B** (data not shown), suggesting that the differential metabolism by the variants was not significantly impacted by growth rate differences.

While the responses of type 2overall were relatively low, this variant clearly outperformed the type 1 variant in the presence of sulfur sources and aromatic amino acid-containing nitrogen sources.. The type 2 variant also generally had a higher response when the peptide nitrogen sources included aromatic amino acids. Interestingly, none of the substrates in plate 5, nutrient supplements, supported metabolism in either variant. **Tables 7 A, B** list the substrates producing the greatest differences in response between the type 1 and 2 variants. More detailed lists of the substrates producing significant differences in response between the variants are provided in **Table S 2, parts A and B**. Though less apparent, the type 1 variant generally outperformed the type 2 variant in the chemical sensitivity assays (Tables 8A,B and Table S-2) with possible greater resistance to certain inhibitory conditions such as pH and antimicrobial substrates such as potassium tellurite (reactive oxygen inducer); membrane-, DNA-, and chemically-active substances; and toxic charged molecules and microbicides. However the overall differences in responses for plates 9-20 of type 1 and 2 variants were less obvious than the metabolic substrate differences, as shown in **FIGURE 6**. Thus these data suggest that the type 1 variant is more adaptable to a broader range of (metabolic input) but in the presence of inhibitors or stressors, the two variants often behave similarly.

Phenotyping of LPS O polysaccharides.

Purified LPS samples from the type 1 and type 2 variants of *B. pseudomallei* MSHR5848 were separated by SDS-PAGE and probed with monoclonal antibody 11G3-1 specific for *B. pseudomallei* LPS. Type 1 and type 2 appeared to be identical in their LPS O polysaccharide (OPS) banding, with a range higher than that of the typical type A LPS banding pattern, as illustrated for *B. pseudomallei* strain 1106a (**Figure 7**). This difference in banding pattern range was also observed using a variety of other monoclonal antibodies specific for *Burkholderia* LPS, as well as by silver staining (data not shown). Interestingly, monoclonal antibody 9D5 was unable to recognize LPS from either type 1 or type 2, signifying that both of these phenotypic variants have OPS structures that differ from typical type A strains. This antibody is specific for a unique conformational epitope found on OPS from certain *B. pseudomallei* strains, and the absence of mAb 9D5 binding indicates alteration of the OPS substitution pattern for both variants (ref - Wikraiphat, C. et al., 2015).

Persister phenotype.

As demonstrated for various bacteria, the *Burkholderia* are thought to produce persister cells which may be a reservoir for chronic infections. These phenotypically altered forms can remain viable in the presence of adverse conditions such those present in the macrophage phagolysosome. To test the type 1 and type 1 variant for possible differences in persister cell formation, an *in vitro* assay comparing their development of tolerance to high concentration of ciprofloxacin was employed, as described previously (ref – Butt, et al.) As shown in **Supplementary Figure 1**, both the type 1 and type 2 variants appeared to produce antibiotic tolerant cells to a comparable extent in cultures treated at early stationary phase with ciprofloxacin but not in those initially exposed to the antibiotic at early log stage. These data suggested that persister cell formation might not be a phenotype controlled by the variant switching that occurs in MSHR5848. However this tentative conclusion requires that the variant responses in assays of persister-associated assays be determined.

Molecular genetic analysis

MLST and rRNA analyses.

Comparisons of the restriction digest patterns for the type 1 and type 2 variants showed that the ribosomal RNA of MSHR5848 was conserved in the variants, as illustrated by the RiboPrints® in **Figure 8**.

Similarly, the sequence type (ST) using the *B. pseudomallei* MLST scheme (**ref. – ?** Godoy, D. et al. 2003) derived from seven housekeeping genes was 553 for all variants (MSHR5848 Solid types 1 and 2 and MSHR5848 Liquid) (data not shown). This ST is consistent with that obtained elsewhere for the strain MSHR5848, according to the mlst.net database.

Whole genome sequence analysis.
To determine whether the phenotypic differences of Types 1 and 2 are based on changes at the DNA level, the WGSs of both were determined. Initial sequencing used gDNAs prepared from broth cultures of single colonies of type 1 and 2 and sequenced on a PacBio RS II. Fourteen sequence differences between type 1 and 2 were identified (data not shown). Sanger sequencing done on ten of the 14 sequences identified two which produced distinctly different PCR products for type 1 and 2 DNAs. Although the variations were not located in regions with annotated functions, further analysis might be warranted.

To verify that the process of growing the cultures in broth did not skew sequencing results, gDNAs were separately prepared from individual colonies of each variant and sequenced directly on the Illumina MiSeq. Three separate sequencing analyses were completed to improve coverage of all regions harboring variant sequence differences and to identify any reproducible findings. For chromosome 1, a total of 30 sequence variants were identified (**Table 8-A**. Fourteen of these variants were in noncoding regions and 16 in coding regions of the genome. One cluster of differences was observed with high confidence in the inorganic pyrophosphatase gene (DP65_RS04090) that resulted in a frameshift and early truncation in the type 1 protein WP_004522531) as shown in **Figure 8**. For chromosome 2, there were 6 sequence differences in noncoding regions and 8 differences in coding regions (**Table 8B**). A second high confidence call was a three base deletion/insertion in a chromosome 2 putative gene (DP65_RS19870) that resulted in a frameshift in a putative protein (WP_038760828.1). This frameshift, which occurred at the 3' of the gene, did not have an impact on the protein itself as the stop codon was still appropriate (data not shown). Further work is required to confirm all identified variants by Sanger sequencing and complete consensus sequence analysis on all regions of the genome. The significance of these WGS results for the mechanism of MSHR5848 type switching will require the construction of mutants and analysis of the impact of the mutations on phenotypic reversion and pathogenicity in animals.

Infection of macrophages and mice.
Mouse challenge.
The virulence of the variants was compared using BALB/c mice challenged by the IP route, a model useful for *Burkholderia* strain comparisons as described previously (ref - Welkos et al., 2015).
Both the day 21 and day 60 LD_{50}s of type 2 were about ten-fold less than that those of type 1, and the day 21 values were significantly different with a probability $\geq$95% by Bayesian probit analysis (**Table 9**). Also, at day 21, type 2 exhibited greater potency at all doses associated with 23% to 92% lethal rates (**Supplementary Figure 2**). Although the lower day 60 LD_{50} of type 2 was not significantly different from that of type 1, type 2 exhibited greater potency at all doses associated with 79% to 93% lethal rates (data not shown).

Macrophage infection.
The ability of the type 1 and type 2 variants to infect and survive and induce cytotoxicity for J774.A1 macrophages was determined. Type 1 was phagocytosed to an almost 5-fold greater extent than type 2, as shown by the viable counts collected after the 1 h uptake and 2 h antibiotic treatment incubations (**Figure 10**). The viable counts recovered from type 1-infected cells at all three time points, to include the final 8 h samples, were greater than those from type 2-infected macrophages ($p<0.0001$). In addition, the type 1 variant was much more cytotoxic for the cells. As shown in the legend of **Figure 10**, type 1 induced much more cell death and cell detachment/loss from wells than did type 2, and was associated with a greater extent of MNGC formation, as evidenced by the larger proportion of MNGCs and the greater mean

number of nuclei within the MNGCs. The greater cytotoxicity and cell loss associated with type 1 suggests that the 8 h viable counts likely underestimated the extent of the intracellular replication by type 1.

DISCUSSION -

Two distinct and relatively stable phenotypic variants were produced by *Bp* strain MSHR5848. These variants, type 1 and type 2, differed widely in a large array of phenotypes to include colony/cell morphology, biochemical sensitivity or utilization, macrophage survival and activity, and animal virulence. These differences could be driven by a heritable mechanism, epigenetic changes in response to an altered environment, or a combination of mechanisms, as will be discussed. In this paper, we characterized the magnitude and breadth of phenotypic changes influenced by this variation. Ongoing studies with MSHR5848 focus on discerning the mechanism for the phenotype switching, the metabolic pathways involved, and the impact of the phenotypic switching on strain pathogenicity and virulence.

Both variant types were present in the source vial and in the two subsequent stocks prepared by the solid medium procedure (**Figure 4**). However the proportion of type 1 increased significantly with each subsequent stock preparation (**Table 1**), an observation likely explained in part by the small but significantly faster *in vitro* growth rate of type 1 compared to type 2 (**Figure 5**). Both of the two major variants were stable upon passage (**Table S-1**); however, both type 1 and 2 were capable of reverting to the alternate morphotype, albeit in a random and stochastic manner. Type 2 reverted more frequently and after exposure to a wider range of *in vitro* conditions than did Type 1 (**Table 3**). Reversion of the latter only observed with type 1 variants freshly isolated from one type 2 single colony stock incubated under one condition (**Table 3**). This disparity in the extents and randomness of variant switching is a common finding and has been reported previously for *Burkholderia* and other organisms capable of phenotypic variation, as described below.

The production of distinct morphological variants from the same strain of *B. pseudomallei* is a well-established observation, and the colony switching of strain MSHR5848 appears to represent a modification of that described previously for *Bp* and related *Burkholderia*. For example, of the two distinct variants reported by Nicholls (ref - Nicholls 1930), the rough one was present in much greater proportion than the smooth variant, in agreement with the proportion of the rougher type 2 and smooth type 1 variants in the MSHR5848 source vial and master seed stocks. However unlike the instability of the mucoid form and its propensity to switch to the rough forms (ref –Nicholls 1930), we observed greater stability and a low incidence of switching for both type 1 and 2 variants grown under typical laboratory conditions (**Table 3**). Nicholls et al. also described a more dense and yellower intermediate form of the smooth form that was similar to a randomly occurring derivative of type 1 and which reverted to the original type 1 morphotype (**Table S-1**). Another similarity was the finding of additional cultural and biochemical characteristics that distinguished the variants (ref - Nicholls 1930), as described here (**Table 2**).

The predominance of the rough dry type 2-like morphology in *Bp* strains cultured from melioidosis patients was confirmed by Chantratita and colleagues (ref –Chantratita et al., 2007). This rough dry morphotype I appeared to be the parent from which the others arose *in vivo* as well as *in vitro*; our findings with the MSHR5848 variants may support these conclusions as shown in **Table 3.** Stressful laboratory conditions promoted switching of morphotype I which progressed with time of incubation and progressive starvation (ref – Chantratita, 2007), as observed in the MSHR5848 work (condition 9, **Table 3**). Despite strain specific heterogeneity in responses to some stressors, evidence from the previous work and our current studies supported the idea that different colony morphotypes may reflect adaptive changes that enhance fitness in a particular environment (ref-Chantratita et al., 2007;Tandhavanant et al., 2010).

Several recent studies have expanded upon the range of strains and phenotypes impacted by phenotypic switching of *Bp*. As reported by Austin et al. (ref –2014), *Bp* strain K96243 exhibited colony variants which differed in morphology as well as other *in vitro* phenotypes, despite their genetic identity.

The white and yellow colony types grew differentially *in vitro* under low oxygen or acidic environments and exhibited several characteristics similar to those of the MSHR5848 variants. For both *Bp* strains, the variants were relatively stable but reversible, the kinetics of morphotype switching varied and was subject to stochastic variation, only the yellow variants produced extracellular DNA, and an association between the *in vitro* phenotypic variation and differential *in vivo* survival was shown (ref- Austin 2015). Only one of the K96243 yellow forms, albeit highly attenuated, could persist in the harsh conditions of the murine stomach and colonize the mucosa. Similarly, MSHR5848 type 1 was less virulent than type 2 for mice by aerosol and IP routes but type 1exhibited greater persistence or resistance to inhibitory conditions, eg., the macrophage environment (**Fig. 10**) and inhibitory chemicals (PM substrates, **Table 7A, B and Table S-2**). Survival in a defined niche such as the gastric mucosa or splenic macrophages may not be predictive of animal survival. Wikraiphat et al. described mucoid (M) and nonmucoid (NM) variants from the same sample which were identical genetically but exhibited antigenically different LPS O polysaccharide (OPS) (ref –Wikraiphat); a conformational epitope appeared to be associated with differing OPS residue substitution patterns. Switching from NM to M type occurred for more pairs and under a larger variety of *in vitro* growth conditions than the M to NM reversion, similar to the findings with MSHR5848 variants (**Table 3**). Furthermore, a greater extent of phagocytosis by macrophages was associated with the M compared to NM variant, again similar to the MSHR5848 variants (**Fig. 10**). Although the regulatory mechanisms are not well understood, the association of colony type and OPS modification might explain some of the colony variation described previously (ref –Nicholls 1930). Lastly, Gierok and coworkers isolated six or eight colony morphotypes, respectively, from *Bp* strains K96243 and E8 after prolonged growth of the strains under nutrient starvation conditions. The morphotypes varied metabolically in nutrient uptake and secretion (carbon sources and amino acids); and in recovery from infected macrophage cultures and from lung samples of mice collected three days post-infection. For instance, all the morphotypes consumed glucose and secreted gluconate but differed greatly in the extent and rate of these activities. Interestingly, intracellular passage of K96243 morphotyes in macrophages and mice selected for isolates with a single morphotype and similar metabolic profiles. This infection-induced reduction in variant types supports the role of environmental stressors in strain switching to maximally fit variant(s) (**ref** – Austin 2015; Chantratita 2007; Vial, L. 2010;;Tandhavanant et al., 2010).

There are several possibilities which could potentially explain the basis for the smooth moist type 1, in contrast to the flatter matte type 2, colony morphologies of MSHR5048. A role for LPS O polysaccharide modifications, as described previously, is possible but unlikely (ref - Wikraiphat et al). Type 1 and 2 produce antigenically identical OPS as determined by a panel of LPS-specific mAbs in this study (**Figure 7**). In addition, antibody 9D5 was unable to recognize OPS from either variant, so it appears both of these phenotypic variants have similarly modified OPS corresponding to a lack of both 4-*O*-acetyl and 2-*O*-methyl groups on the 6-deoxy-L-talose residues (**ref** - Wikraiphat et al). Secondly, a role for the *Burkholderia* polysaccharide capsule (CPS) may be feasible. The CPS expressed by the human *Bm* and *Bp* isolates is surface—associated, a proven virulence factor which imparts resistance to complement – mediated killing and to neutrophil phagocytosis and killing, and induces protective anti-CPS antibodies (ref. – Deshazer Mic Pathog 2001; Reckseidler-Zentano IAI 2005;Sarkar-Tyson JMedMic 2007; Woodman, PLoSOne 2012; Cuccui, IAI 2012; Jones, S.M. JMedMicro 51:1055, 2002; Nelson, M. JMedMicro 53:1177,2004; Parthasarathy, N., Diagn.Microbiol. Infect.Dis,56:329, 2006; Cuccui, IAI 2012). Thirdly, as discussed below, a mechanism similar to that influencing phenotypes such as colony color and gastric colonization is possible (ref – Austin 2014). Although the environmental conditions inducing reversion of the K96243 variants differed from those we described for the MSHR5848 variants, for both strains, reversion frequencies varied stochastically and only the MSHR5848 type 1 was associated with surface or secreted DNA (ref – Austin 2014). Studies are being pursued to further characterize the MSHR5949 variant differences in CPS, LPS and DNA secretion in *vitro* and *in vivo* to better understand the basis of their distinct colony morphologies.

Although the phenotype reversion of MSHR5848 appeared to generally resemble the behavior and stability described previously for other *Bp* strains, MSHR5848 colony variation was accompanied by alterations in a more diverse array of phenotypes than reported previously (**ref** - Wikraiphat 2015;

Nicholls, 1930; Austin 2015; Gierok 2016), and as summarized in **Table 2**). The broad and pleiotrophic nature of the phenotypes impacted by MSHR584 type switching suggests a role for a master regulatory locus(s) with global control over multiple genes or regulons (discussed below). For instance, the MSHR5848 type 1 and 2 variant responses to the substrate phenotypes screened by the PM system (**Tables 7A, 7B, and S-2**) suggested that type 1 was metabolically more active in response to a broader range of potential carbon sources than type 2, whereas as type 2 preferentially utilized various substrates as sole S or N sources (**Tables 6, 7A, 7B, S-2;** and **Figure 6**). Given the complexity and heterogeneity of isolates from niches which are themselves complex and evolving, such as the infected CF lung as illustrated above, it is challenging to interpret the potential *in vivo* significance of the in vitro responses. Nevertheless the metabolic response patterns are likely involved in niche adaptation and a coordinated regulation of metabolism and virulence which promotes pathogen infectivity. Metabolism is increasingly being recognized to intimately interwined with pathogenesis (ref – Kwaik, 2013; Rohmer, Trends Mic 2011; Sperandino,2015; Richardson MicroSpec 2015; Somerville 2009; Conway-Cohan, book 2015). To exemplify this potential role in infection of "nutritional virulence", the ability of Type 1 variant to utilize a greater diversity of carbon sources (PM plates 1 and 2) could conceivably lead to an initial growth advantage when exposed to nutrient-rich niches such as the intestinal tract or infected lungs. For instance, type 1 preferentially used the amine compounds putrescine and carnitine. Various gram-negative bacteria have similarly been shown to metabolize these polyamines as sole C and/or N sources (ref - Kleber, H.P. FEMS Microbiol Let 147:1-9, 1997; Shah, MolMicro 2008). They and other polyamines are widely distributed and are essential for normal growth and multiplication of both prokaryotic and eukaryotic cells. Carnitine contributes to fatty acid transport into mitochondria, and also has important roles in protection from osmotic stress and temperature extremes (ref - Kleber, 1997; Meadows, J.A. et al. 2015. Microbiol 161:1161-74). Such activity might contribute to the greater resistance of type 1 to inhibitory substrates, as discussed below.

Despite a possible initial growth advantage of type 1 in nutrient-rich niches, the evolving bacterial adaptation and deregulation of the utilization of redundant carbon sources could lead to loss of metabolic pathways not required for growth in exchange for the expression of virulence factors required for enhanced chronic persistence and virulence of type 2 (Ref – Vial 2010, Cullen 2015, Price 2013, Yang 2011, Bernier 2015). The less metabolically active type 2 was more virulent as determined by the nearly unchanged LD_{50} values measured at acute (day 21) and chronic (day 60) times post-exposure by the IP and aerosol routes (**Table 9** and Soffler, C. and Waag, D, unpublished). In addition to *Bp* strains such as MSHR5848, members of the Bcc exhibit phase switching in phenotypes associated with their ability to colonize the lungs, form biofilms, and cause severe infection in CF patients as opposed to their adaptation to environmental niches such as the plant root (ref – Vial et al. 2010; Bernier et al. 2008.IAI 76: 38–47; Bernier_NatGen 45-5_2014; Goven, JP.2007. Future Microbiol 2: 153–164).

The type 2 variant clearly outperformed type 1 in sole dipeptide nitrogen and sulfur source utilization. The capacity to import and utilize small peptides is common in many bacteria (ref -Matthews, D. et al. 1980. Transmemb. Transport of small peptides. In: Curr Top in Membrane and transport. New York: Academic press, Vol. 14, 331-425 [=Peltzer ref #8]). They can serve as nitrogen and carbon and expand the nutrients available in harsh environments where nutrients are scarce. The host cell is limiting for free amino acids but has other molecules which can be degraded, producing oligopeptides sources of amino acids (ref – Peltzer 2014; Conway, Cohan 2015 book; Abu Kwaik 2013; refs from Kwaik – Alkhuder 2009; Meibom 2010; Al-Quadan 2012; Price 2011; Niu 2008; Niu 2012; Ouellette 2011). PM analysis of substrates preferentially utilized by the Type 2 variant included 16 peptides in the PM 7 sole nitrogen/dipeptide plate (**Table 7B** and **Table S-2**). Thus type 2 might be especially efficient in the uptake or use of such peptides, and their metabolism indicates that MSHR5848 has systems to transport peptides and potentially generate amino acids from them. *Bp* and *Burkholderia mallei* (*Bm*) have numerous ABC transporter homologues, to include some known to transport oligopeptides (OppABCDF) and other proteins (refs – Garmory, H. and R. Titball. 2004. Infect. Immun. 72:6757–6763; Holden, M.T.G. 2004. PNAS 101:14240; Harland, D. 2007. BMC Genomics 8:83; Harland. D. 2007. IAI 75:4173-4180; Lassaux, P. 2013. Structure 21:167-175). Of interest, the type 2 variant was especially

active in their respiration of dipeptide substrates containing aromatic (aro) amino acids, to include tryptophan, phenylalanine, and/or tyrosine (**Tables 7B and S-2**). These molecules play significant roles in the biosynthesis and activity of microbial siderophores, which chelate and specifically bind iron and deliver this essential nutrient to the bacteria via specific surface receptors (ref. – Telford, J. JBIC 2:750_1997; Drechsel, H. JBact 1993; Neilands JBact 1995; Ghosh, J. Mol Mic 2004). They are essential for bacterial survival in the iron-limiting conditions in the host, where the already low iron concentrations are accentuated by serum transferring and lactoferrin (ref. – Telford1997; Drechsel, 1993). The pathogenic *Burkholderia* have multiple siderophore and hemin/hemoglobin utilization systems and inner membrane transfer proteins available for the acquisition of iron in vivo (ref - Wiersinga, W. NatReviews 4:272_2006; Kvitko et al. 2012 PLoSNegTropDis; Alice JBact 2006; Yang, H. IAI 61-656_1993; Biggins, J. J.Am Chem Soc. 2012_134-13192; Mott, PLoSNegTropDis 2015; Kvitko et al. 2012 PLoSNegTropDis; Braun V. FEMS Microbiol Rev. 1995; 16: 295–307). The more efficient mobilization of such systems by type 2 might allow it to better utilize aro amino acid –containing substrates to promote its survival in iron-limiting host niches.

Sulfur and sulfur-containing compounds are essential for *in vivo* growth and persistence of *Bp*, but they and other nutrients vary greatly in availability depending on the niche (eg., bloodstream, host cell, macrophage phagolysosome, etc.) and on inflammatory response-induced adverse conditions such as hypoxia and acidity (refs - Kwaik; From Isolates MSS ref: Mulye et al., 2014; Dowling, A. 2010; Loprasant, S. 2003; Tandhavanant S, 2010; Valvano 2005; Utaisincharoen P, 2001). The retrieval by pathogens of amino acids scavenged from host-degraded protein is a critical adaptive response. It is especially important for amino acids which are essential but relatively scarce, with cysteine being the most limiting amino acid (refs - Kwaik, Bumann). The strategies used are far-ranging. For instance, *Legionella* facilitates proteasome degradation of ubiquinated proteins to access the amino acids and other breakdown products (ref- Al-Quadan 2012; Price 2011 [Kwaik ref.]); *Anaplasma* spp. triggers the degradative process of host autophagy (ref – Kwaik; Kwaik refs Niu 2008; Niu 2012); *Coxiella* and *Chlamydia* exist in phagocytic cell lysosomes where they leverage host lysosomal degradative enzymes to provide source of amino acids (ref - Ouellette 2011 [Kwaik ref.]); and *F. tularensis* resides in the host cell cytosol and has the ability to use cysteine- or cystine-containing sources as well as thiols such as glutathione (ref- Alkhuder 2009; Meibom 2010 [Kwaik ref.]). Thus the enhanced capacity of Type 2 to utilize a variety of peptide and cysteine sources such as cysteine containing dipeptides (PM7) and the tripeptide glutathione (GSH) and related thiols cystathionine and L-anthionine (PM4) (**Table 7B and Table S-2** list) could promote its survival in amino acid and sulfur limiting environments.

Intracellular Bp can leverage host cytosolic signals to upregulate virulence genes. Wong et al showed that Bp can sense the small sulfur-containing thiol GSH through its type six secretion system (T6SS) membrane histidine sensor kinase VirA as a signal to upregulate virulence genes. After its escape from the phagolysosome, the T6SS is activated in the cytosol where it mediates cell fusion and intercellular spreading. In host cells which were chemically depleted of GSH, T6SS activation and MNGC formation was significantly reduced. The conversion of methionine to cysteine is a key earlier steps in thiol synthesis in host cells. These were among sulfur compounds more highly used as S sources by Type 2 than by type 1 (**Table 7B and Table S-2**) and might facilitate this virulence-promoting process. Finally, the high concentrations of N and O reactive species often generated in host oxidative responses to infection can induce changes such as the oxidation of thiosulfate to tetrathionate (ref – Rohmer review. Trends Micro 2011). Tetrathionate inhibits growth of many gram-negative bacteria, although certain pathogens to include *Salmonella* can use tetrathionate as an energy source since it's an electron acceptor for anaerobic respiration (refs – Rohmer, Trends Micro 19-341_2011; Kwaik. 2013. Cellular Micro). Similarly, type 2 was more metabolically active than type 1 for the sulfur-containing compounds tetrathionate and thiosulfate substrates in PM plate 4 (**Table S-2**). Conversely, these thiols might represent stressors which can induce type 2 to revert to the less responsive type 1 in situations where alternate S sources are available; in such circumstances, type 1 may compete better than type 2 for the available carbon as suggested by results of the PM plates 1 and 2 (ref – Boucher. Gen Res, 2001; Zhou, L., Bochner, B.et al, 2003). To examine potential roles these substrates have in morphotype

reversion, preliminary studies were done to assess the extent to which growth in selected PM substrates promoted variant phenotypic switching. The results of growth in the **Tables 7A and 8B** substrates confirmed that both variants were capable of reversion *in vitro*, and suggested that exposure to environments limited in specific nutrients or to antimicrobial compounds might stimulate variant switching *in vivo* (data not shown). Reversion varied stochastically between experiments, however type 2 was capable of reverting to type 1 in the presence of thiol sole sulfur sources (cystathionine, lanthionine, and glutathione).

The responses of the MSHR5848 variants to the extensive array of inhibitory compounds and conditions tested in the PM plate (PM 9 – 20) suggested an overall more extensive resistance of type 1 compared to type 2, as detailed in **Table S-2**. A total of 50 of the 59 substrates which type 1 preferentially metabolized or resisted consisted of inhibitory conditions of PM plates 9 – 20. In contrast, only 13 of the 47 substrates/inhibitors to which type 2 had a stronger response than type 1 were inhibitors (**Table S-2**). These differential responses may have implications in infection. For example, type 1 was more metabolically active under low pH conditions than type 2, resembling the greater persistence of the K96243 yellow variant (compared to the white) in the acidic conditions of the murine gastric mucosa (ref – Austin). Although type 1 was reduced in systemic virulence for mice compared to type 2 (again resembling the K96243 morphologic variant differences), the greater resistance of type 1 to acidic and other toxic host conditions may partially explain its greater infectivity and cytotoxicity in a macrophage model (**FIGURE 10**). Nitrogen and oxygen free radicals and other toxic ions, peptides and enzymes are produced during inflammatory responses to infection (refs. – From Isolates MSS: Mulye et al., 2014; Dowling, A. 2010; Loprasant, S. 2003; Tandhavanant S, 2010; Valvano 2005; Utaisincharoen P, 2001). Bp often localizes to the host macrophage where it must be able to resist the antimicrobial substances (illustrated in **Table 7A** and **Table S-2**) which comprise the phagolysosomal defenses.

For example, potassium tellurite, included in PM inhibitor plate16, is toxic to bacteria due to the generation of superoxide radicals leading to intracellular oxidative stress (ref – Chasteen, T. FEM Mic Rev 2009; Fuentes, D. JBact 2007). Maintenance of the cell oxidation-reduction balance is a critical process and is achieved by molecules made from various reduced environment sulfur sources, especially GSH. A protective effect of GSH against oxidative stress has also been described for other bacteria (refs Fuentes, D. JBact 2007;17, 18 [refs from Fuentes, D. JBact 2007]). Although the type 2 variant was more metabolically active in the presence of sulfur sources in the PM to include GSH, it was comparatively more sensitive to tellurite than was type 1. Little is known about the mechanisms of resistance to tellurite, but it's conceivable that type 1 expresses a mechanism conferring greater resistance to tellurite than does that expressed by type 2. At least five tellurite resistance operons have been identified in Gram-negative bacteria (ref - Chasteen FEMS Mic Rev 2009). Of interest, at least one of these resistance systems involves a membrane protein which imparts resistance to antiseptics and disinfectants similar to that conferred by multidrug resistance efflux pumps (ref – Turner, DE. 1999, Trends Micro 7:111-115).

Type 1 also exhibited greater metabolic activity in the presence of various other antimicrobial cations, anions, mutagens, chelators, reducing agents as detailed in **Table 7A and S2**. These responses again may model exposure to adverse host environments, ie., extra-cellular inflammatory foci and the infected macrophage phagolysosome compartment. For example, thallium acetate and cadmium chloride (PM 13 and 14) are toxic cations which suppress growth of a wide range of prokaryotic and eukaryotic organisms, and the former has long been used in selective media (refs - Conerly O, Rieth S. Toxicological review of thallium and related compounds (CAS No. 7440-28-0). Washington, DC: U.S. Environmental Protection Agency, 2009; Fazeli M, Hassanzadeh P, Alaei S.2011. Cadmium chloride exhibits a profound toxic effect on bacterial microflora of the mice gastrointestinal tract. Hum Exp Toxicol.30:152-9). Hydroxyquinolone (PM20) is a strong chelator for metals such as iron and metals necessary for the catalysis of DNA biosynthesis (ref. Shen, A.Y., Chen, C.P, Roffler, S. 1999. A chelating agent possessing cytotoxicity and antimicrobial activity: 7-morpholinomethyl-8-hydroxyquinoline. Life Sci. 64:813-825.) Finally, the compound chloroxylenol (PM plate 16) has disinfectant activity which elicited a more resistant response by type 1 than type 2 (Table 8A and 8 B). As part of the preliminary studies described above to assess variant reversion in the presence of PM compounds (**Table 7A, 7B**),

type 1 showed a variable but high degree of reversion (50%) to type 2 when grown in choroxylenol. Although the type 2 cells were inhibited in growth, revertants were not observed and the inoculum remained viable. In contrast, type 1 bacteria were better able to initiate growth but were susceptible to chloroxylenol-mediated killing; and the remaining colonies included equal numbers of type 1 and type 2. Thus perhaps the stress imposed by the chemical induced reversion of type 1 to type 2 at a high rate. More studies will be needed to assess the role of *Burkholderia* metabolism and genetically-encoded resistance to these and other antimicrobials.

The greater resistance of type 1 to antimicrobial entities such as potassium tellurite (**Table 7A and 7B**) suggests that type 1 might grow initially to a greater extent than type 2 in harsh environments such as the macrophage phagolysosome. As expected from the PM results, type 1 replicated to a greater extent and was more cytotoxic than type 2 for macrophages as illustrated in **FIGURE 10**. Nevertheless, residence in the phagolysosome is a transient phase in phagocyte infection for virulent Bp, which can subsequently escape to the cytoplasm where they mobilize actin, spread to other cells, and facilitate MNGC formation; these functions are perhaps better carried out by type 2, as discussed above. Thus as implied by these findings of differential variant susceptibility, the in vitro findings cannot be directly translated to *in vivo* activities or *Bp* virulence. Nevertheless, these examples support the concept that pathogens adapt metabolically to environmental niches which differ in the extent and nature of available metabolic substrates and in antibacterial stringency in a manner which allows them to evolve in response to the evolving disease. The interactions might result in either chronic persistence of the bacteria, or increasing resistance (refs. - Price et al. 2013. mBio 4:e00388; :Yang et al PNAS 2011; T. Conway, P. Cohen book 2015; Rohmer Trends Micro 2011; Cullen 2015; Lieberman, T.D. 2011. Nat Genet. 43:1275)

Our studies with cell culture and murine infection models revealed that the *in vitro* phenotypic variation of MSHR5848 impacted infection parameters. In contrast to the reduced virulence of type 1 compared to type 2 for mice (ie.,10-fold higher LD_{50}), the type 1 variant was "virulent" for macrophages. These observations agree with the inverse relationship between *in vitro* macrophage and *in vivo* virulence observed for several *Bp* strains (ref -Welkos et al., 2015). This relationship has been observed for other bacterial pathogens, as exemplified by the *Yersinia* pathogens. Although *Y. pestis* (Yp) is the etiologic agent of highly lethal pneumonic plague, it exhibits limited capacity to induce cytotoxicity in macrophages, due in large part to ineffective translocation of its apoptosis inducing TTSS effector protein YopJ (ref -Zauberman et al. PLoSONE 2009). YopP, the YopJ homologue of *Y. enterocolitica* (Ye), is translocated more efficiently and consequently Ye is more cytotoxic than Yp. When YopJ is replaced with YopP in Yp, the YopP-producing Yp is much more cytotoxic for macrophages, but is avirulent; this loss of virulence was associated with its activation of a more aggressive and protective immune response than was induced by the wild type Yp strain (ref -Szaba et al, IAI 2009). Analogously, an inverse relationship was observed between virulence and the presence of an alternate form in Yp of LPS (*lpxl*) which was better able than the wild type LPS to interact with and stimulate cytokine producing T cells. This leads to a greater immune response and a reduction in virulence compared to wild type strains which produce the typical poorly stimulating LPS. A similar association between production of alternate forms of LPS and increased attenuation was described for the enteropathogen *Y. pseudotuberculosis* (ref. - Sun, W. et al. Curr Pharm Biotech 2013; Pujol, Bliska -2003 IAI and 2005 PNAS). Thus the reduced ability of pathogenic strains of *Yersinia* to stimulate or damage host cells may allow the pathogen to multiply within a shielded *in vivo* niche such as a macrophage and more readily avoid immune responses.

The molecular mechanism of the phenotypic variation of MSHR5848 is unknown but is likely not explained by a structural gene deletion. The broad, pleiotrophic, and reversible nature of the phenotypes impacted by the variant switching suggests a role for a master regulatory locus (loci) with global control over numerous genes. Whole genome sequence comparisons of type 1 and 2 confirmed that the variants are genetically relatively conserved. Nevertheless, numerous sequence differences between type 1 and 2 in both chromosomes were identified, with one of the most noteworthy being in the inorganic pyrophosphastase-encoding region NCBI WP_004522531.1). These enzymes hydrolyze inorganic pyrophosphate (PPi) to two molecules of orthophohphates (Pi). Processes such as proliferation, cell survival, mobility, and metabolism are reversibly controlled by protein phosphorylation, which requires

both protein phosphatases and kinases (**refs** - Dick, C.F. et al, 2011; Lahti, R._PPi_1983. Microb.Rev 47:167). Several essential phosphatase enzymes are required for homeostasis of Pi during its acquisition, storage, release, and metabolic incorporation. These enzymes and their activities are regulated via the Pi signal transduction pathway (PHO) in response to varying Pi levels (**ref** - Dick, C.F. et al, 2011. Enzyme Research. Art 103980). Phosphate signaling occurs in various eukaryotic organisms and bacteria and has been connected to pathogenesis (**refs** - Dick, C.F. et al, 2011; Larache, M.G. 2008, FEMS Microb.Rev. 32:1-13; Bliska, J.B. 1 991. PNAS 15:1187). The specific role of the pyrophosphastase identified here in the colonial and other phenotype differences of the variants, if there is one, is not yet known. Studies are being pursued to confirm the prevalence of all the potentially significant sequence variations (**Tables 8A,B**) and to identify the impact of mutations in targeted sequences on the phenotypic diversity of MSHR5848.

A transcriptional regulator controlling colony color variation was identified in the studies of Austin et al (ref - 2015). This regulator, BPSL1887, encoded by *BPSL1887* (or *yelR*), is also linked to expression of several other in vitro phenotypes. It remains to be determined whether the *yelR* gene, multiple genes with different mechanisms regulating phenotypic diversity, or a master switch controls this and other reversible phenotypes described for K96243 and other *Bp* strains. These authors hypothesized that a master regulator, in the form of a bistable switch, may be involved in the regulation of colony color and other phenotypes characterized for K96243, as described previously by Dubnau et al. (ref – Austin 2015; Dubnau, D. et al 2006 Bistability in bacteria. Mol Micro 61:564-572). Although the overarching mechanism responsible for phenotypic diversity remains undefined, the production by many *Bp* strains of reversible variants with striking differences in several phenotypes in the presence of environmental stressors suggests that post-transcriptional or -translational events are the immediate inducers of the phenotype switching. Similar immediate epigenetic events possibly also drive the switch in the LPS O-PS substitution pattern, and the *in vitro* and *in vivo* reverting *Bp* phenotypes described by others (refs - Wikraiphat IAI 2015; Gierok 2016; Chantratita et al.,2007; Chantratita et al., 2012; Velapinato et al 2012). Finally, the mechanisms of phenotypic variation determined for unrelated bacterial systems may be informative. For example, the capacity of the pneumococcus (*Streptococcus pneumoniae*) to reversibly switch between opaque and translucent colony types is a long established example of reversible phase variation (ref - Wisniewski-Dye and Vial_2008; Manso, A. 2014 Nature Comm.). Manso et al. showed that genetic rearrangements in a restriction/modification system mediated epigenetic modifications which impacted both the colony phenotype and virulence (ref - 2014, Nature Comm.). The six variant subpopulations identified exhibited distinct gene expression profiles and differences in virulence as shown by *in vivo* switching between variants.

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Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National

Research Council, 2011. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

BURK 178 manuscript – FIGURES

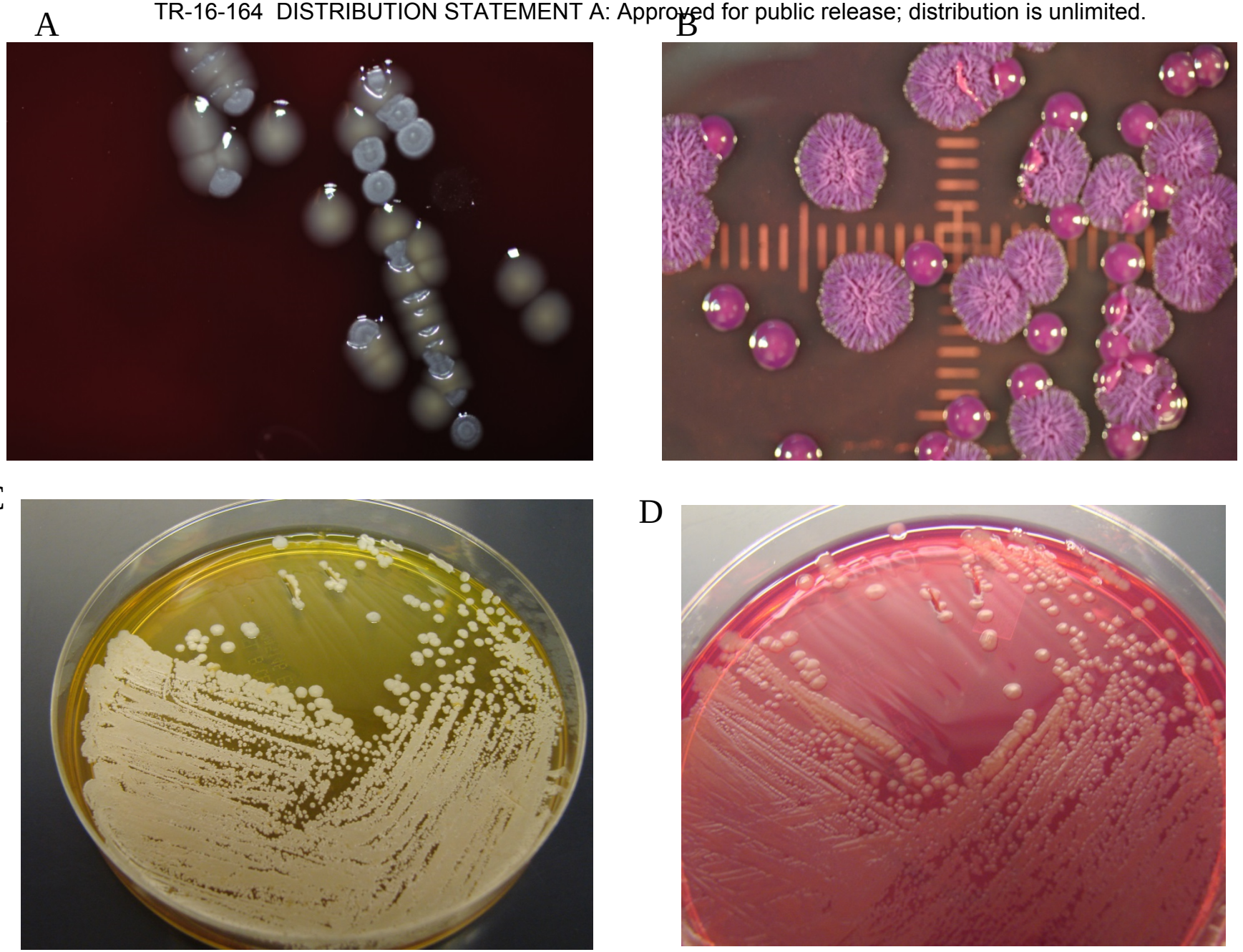
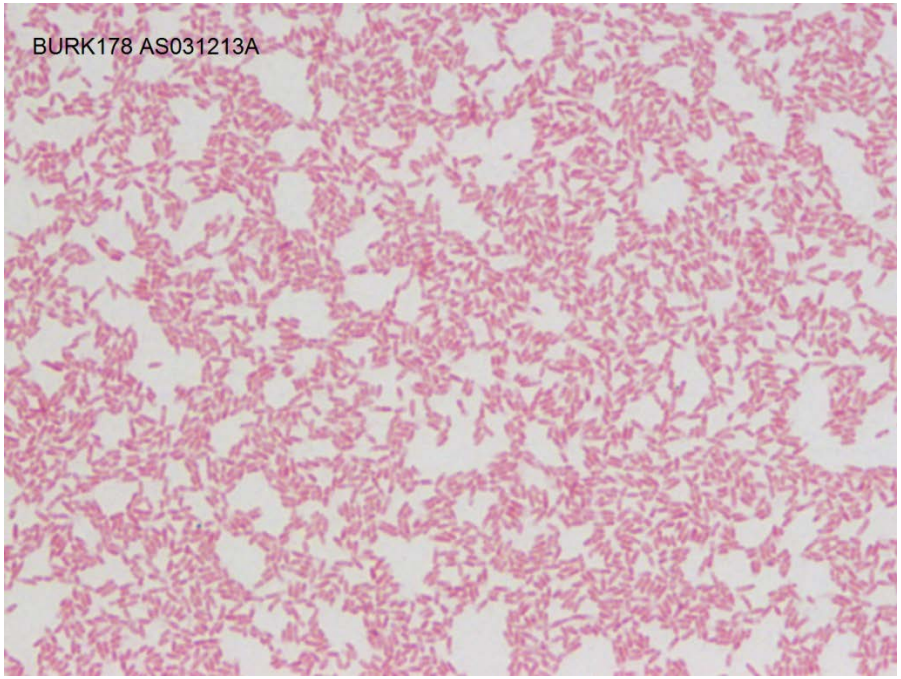


Fig. 1. Type 1 and 2 variant colony morphologies. A – Sheep blood agar plates. B – Ashdown's agar plates. C and D – BSCA plates with type 2 (lactose-positive) and type 1 (lactose-negative), respectively.

A



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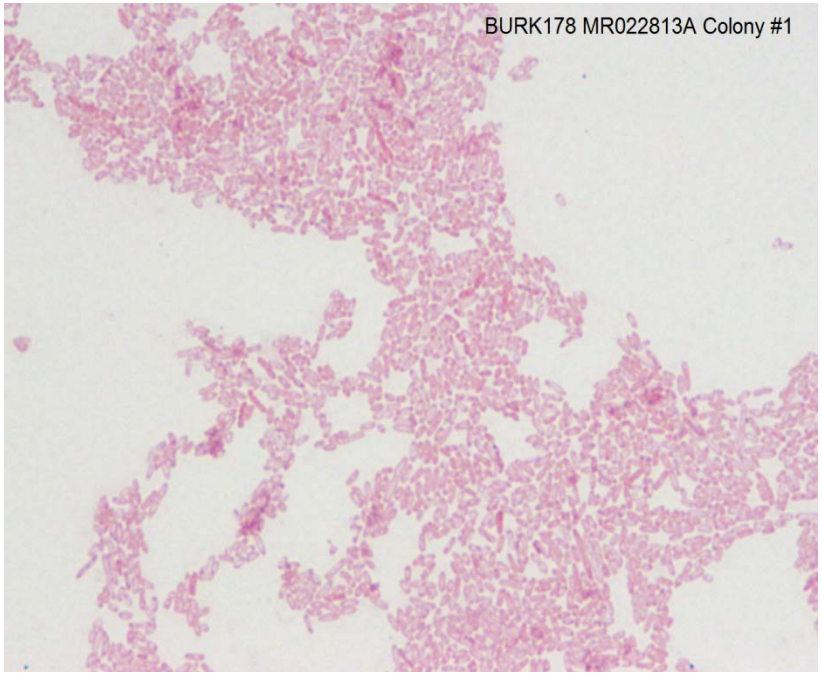

Fig. 2. Type 1 and 2 microscopic morphologies. A, B – gram stains of type 1 and 2, respectively.

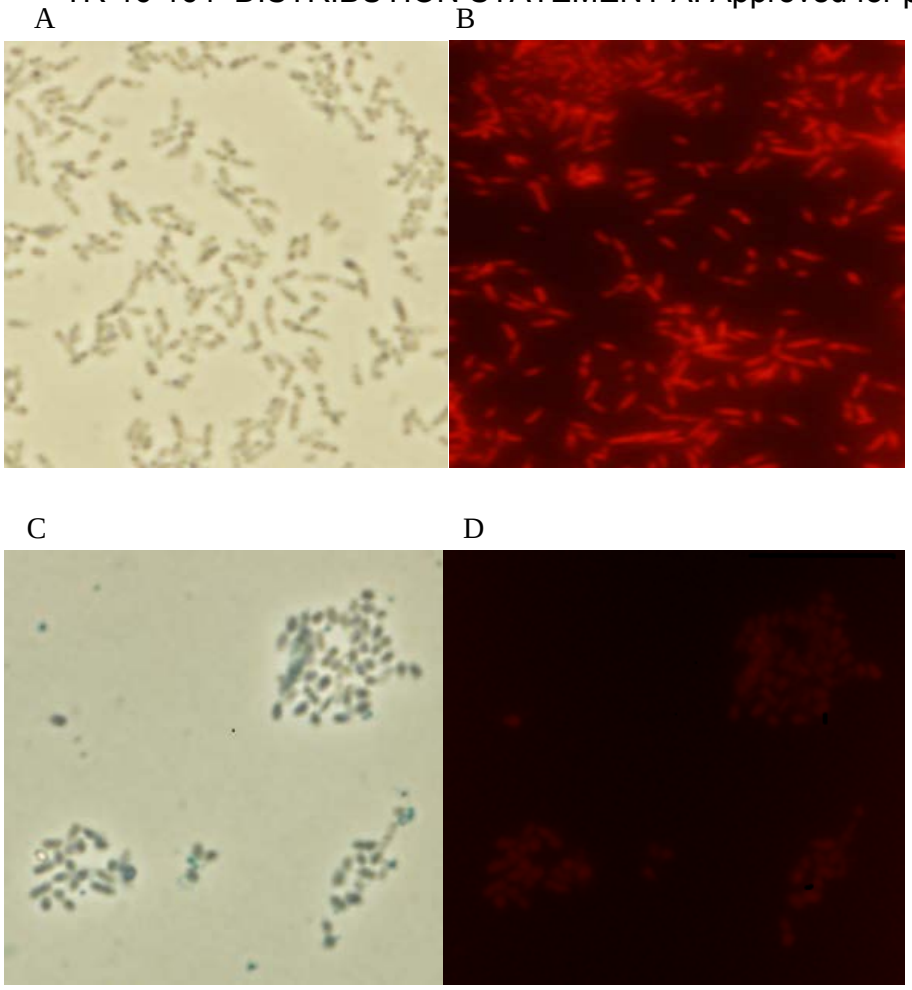

Fig. 3. Staining of bacteria with propidium iodide (PI). The MSHR5848 solid stock was grown on SBA plates at 37C for three days. Type 1 or type 2 colonies were suspended in PBS and the suspensions were dried on microscope slides and stained with the fluorescent DNA binding dye PI. The slides were viewed on an Olympus BX51 microscope with phase contrast (100x, oil immersion objective) or fluorescence (exc 535 nm/ em 617 nm) microscopy. A – Type 1 colony bacteria viewed by phase contrast. B – Type 1 colony bacteria stained with PI and viewed with fluorescence. C - Type 2 colony bacteria viewed by phase contrast. D - Type 2 colony bacteria stained with PI and viewed with fluorescence.

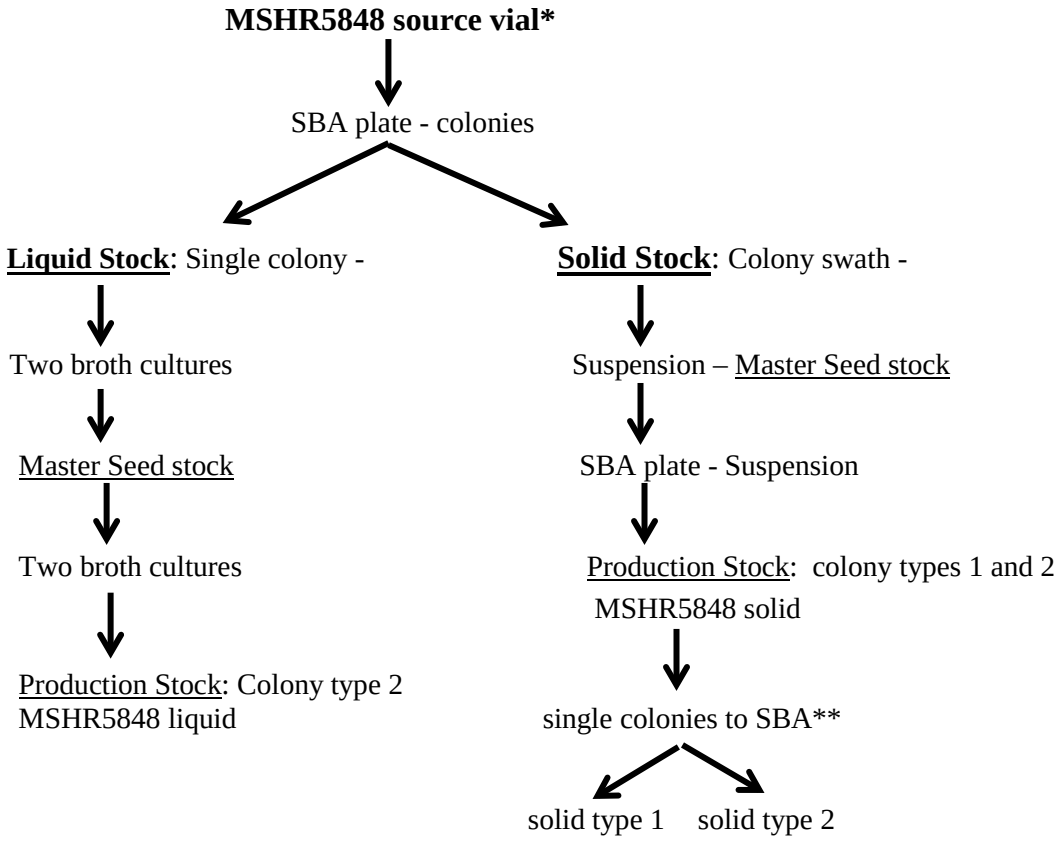

Fig. 4. Derivation of strain stocks of *Bp* strain MSHR5848.
* *B. pseudomallei* strain MSHR5848, a human clinical isolate from Australia (Menzies School of Health Research), was received by and maintained in the USAMRIID Uniform Culture Collection (UCC) as stock BURK178.
**Subculture of individual type 1 or type 2 colonies produced cultures that were essentially non-reverting under typical growth conditions. Solid or liquid cultures of them consistently produced colonies > 99% of which was the single morphotype.

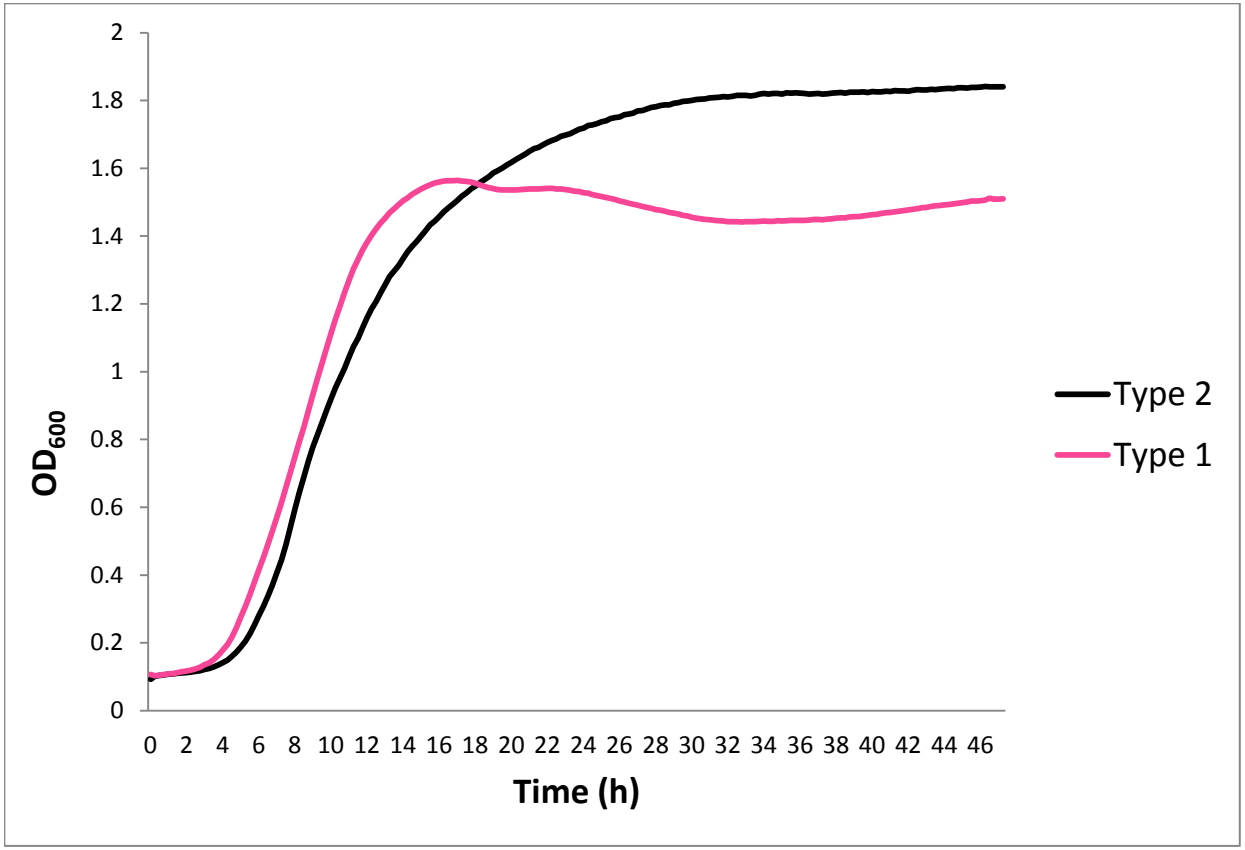

FIG. 5. Growth in broth culture of MSHR5848 type 1 and type 2. Growth curves were determined for type 1 and 2 colonies isolated from the Master seed stock using the Bioscreen C instrument. Type 1 multiplied at a higher rate and reached stationary phase before type 2. These curves duplicate those measured for type 1 and 2 colonies derived from the Production stock.

The two variants had differential substrate utilization and antimicrobial resistance patterns

Fig. 6. Phenotypic Microarray data. The 20 PM plates are used to identify strain differences in ability to metabolize different sources of carbon, nitrogen, phosphorus or sulfur; or differences in sensitivity to antimicrobial conditions. Kinetic data were collected every 15 min for 48h and the variant curves compared, as shown. **Gray** – overlapping activity, **rose** area - greater type 1 response, and **black** area - greater type 2 activity.

Type 1 was overall metabolically more active (eg., in the presence of sole C sources, PM 1, 2) and resistant to some antimicrobial conditions; but type 2 outperformed type 1 in the metabolism of sole S sources (PM 4), aromatic dipeptide N sources (PM 7) and in responses to some inhibitors.

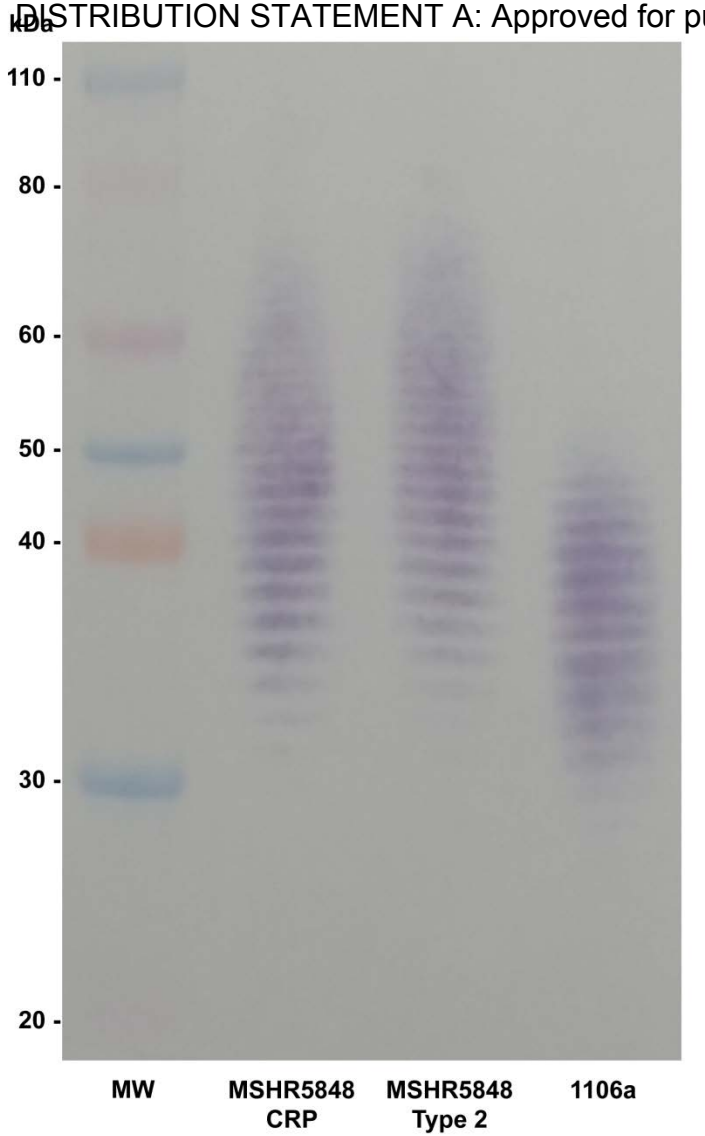

Fig. 7. Western blot of LPS from *B. pseudomallei* strains. Purified LPS from MSHR5848 type 1 and type 2, and *Bp* strain 1106a, were separated by SDS-PAGE and a western blot was done using monoclonal antibody (mAb) 11G3-1, specific for *B. pseudomallei* LPS O polysaacaride (OPS). Unlike the typical Type A banding pattern of most *Bp* strains (1106a), the variants displayed identical higher molecular weight patterns.

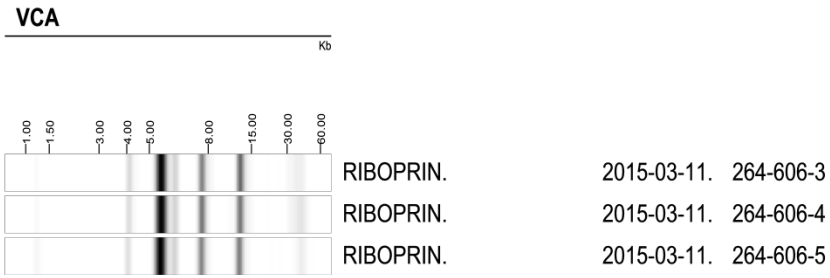

Fig. 8. Riboprints of type 1 and type 2.

Fig. 9 - Whole genome sequence comparisons: Potential phenotype to genotype linkage for MSHR5848 variants. Consensus sequences for type 1 and type 2 were aligned to identify significant sequence variations (orange) that could account for the differences in observed phenotypes. A two nucleotide insertion (left box), within a 12-base region with multiple differences) in the type 1 inorganic pyrophosphatase gene (DP65_RS04090) resulted in a frameshift with a premature stop codon (right box). This results in a premature truncation in this protein (94 amino acids vs 175).

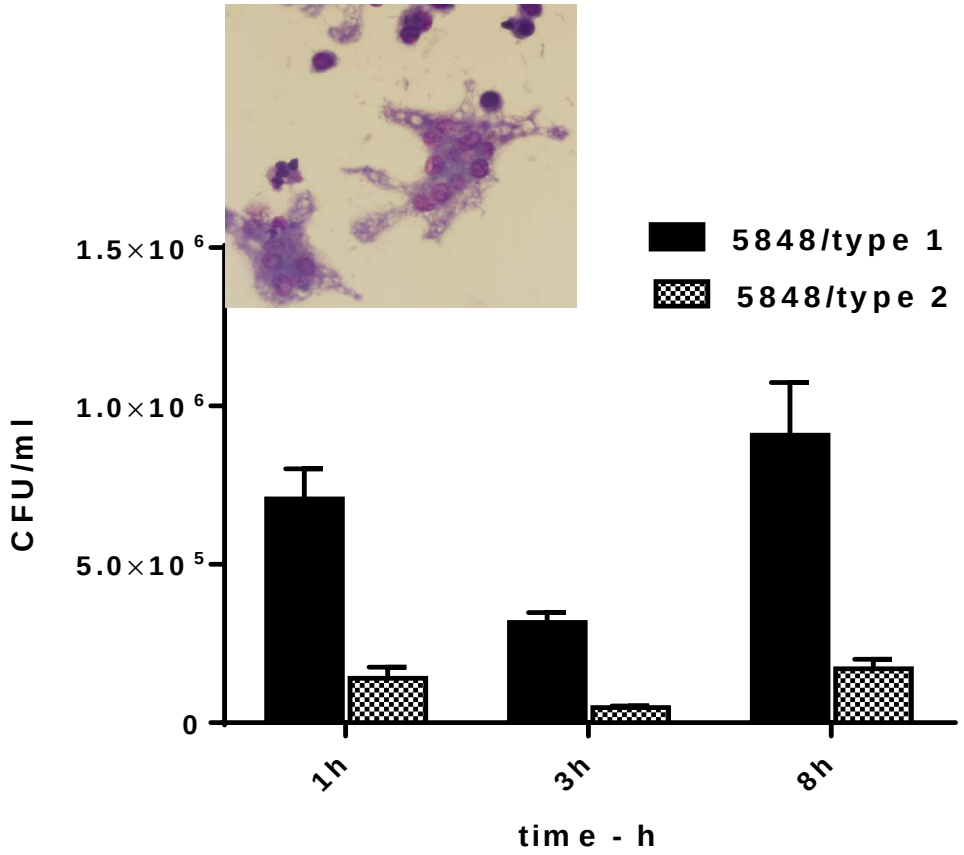

Fig. 10. Infection of J774.A1 macrophage cultures with two variants of *B. pseudomallei* MSHR5848[a], type 1 and type 2. The MOIs were 19.4 and 19.1, respectively. The 5848/ type 1 was phagocytosed to an almost 5-fold greater extent than the 5848/ type 2 strain, as shown by the 3h viable counts. The counts recovered from type1-infected cells at all three time point were greater than those from type 2-infected macrophages ($p<0.0001$). Also, much greater cell death/detachment and MNGC formation was associated with type 1 in samples collected and stained at 8h, as shown in the table:

Strain	% cell loss	%cells dead (TB)[b]	MNGC or necrotic cells (% of total)[c]	MNGC nuclei (%)[c]
5848/type 1	75	75	17.7	67.2
5848/type 2	15	12.5	3.6	14.5

[a]Referred to as BURK178/CRP in Unified Culture Collection.
[b]TB - determined by trypan blue staining and does not include unstained necrotic (dead) cells.
[c]The extensive cell loss induced by 5848/Solid infection minimized the significance of these values.

Supplementary Figures

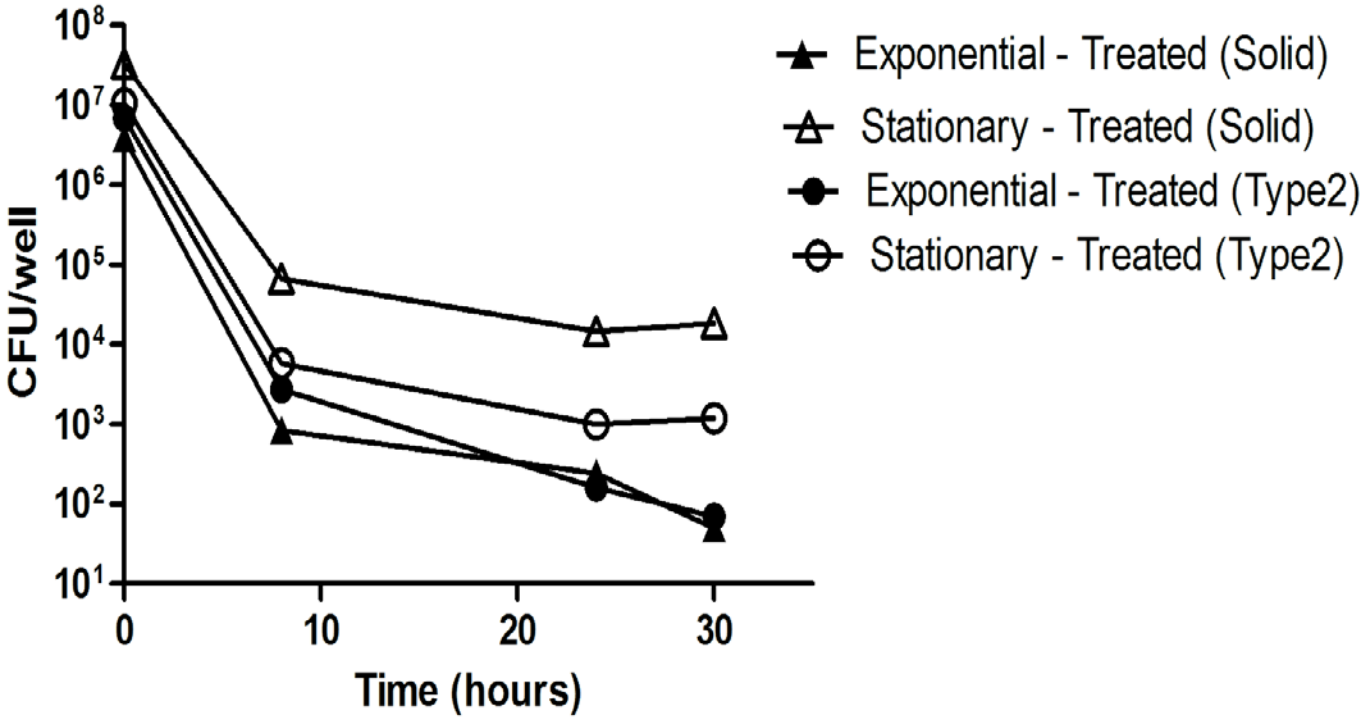

Fig. S-1. Persister phenotype. Both the type 1 and type 2 variants produce antibiotic tolerant cells to a comparable extent in cultures treated at early stationary phase with ciprofloxacin but not in those initially exposed to the antibiotic at early log stage.

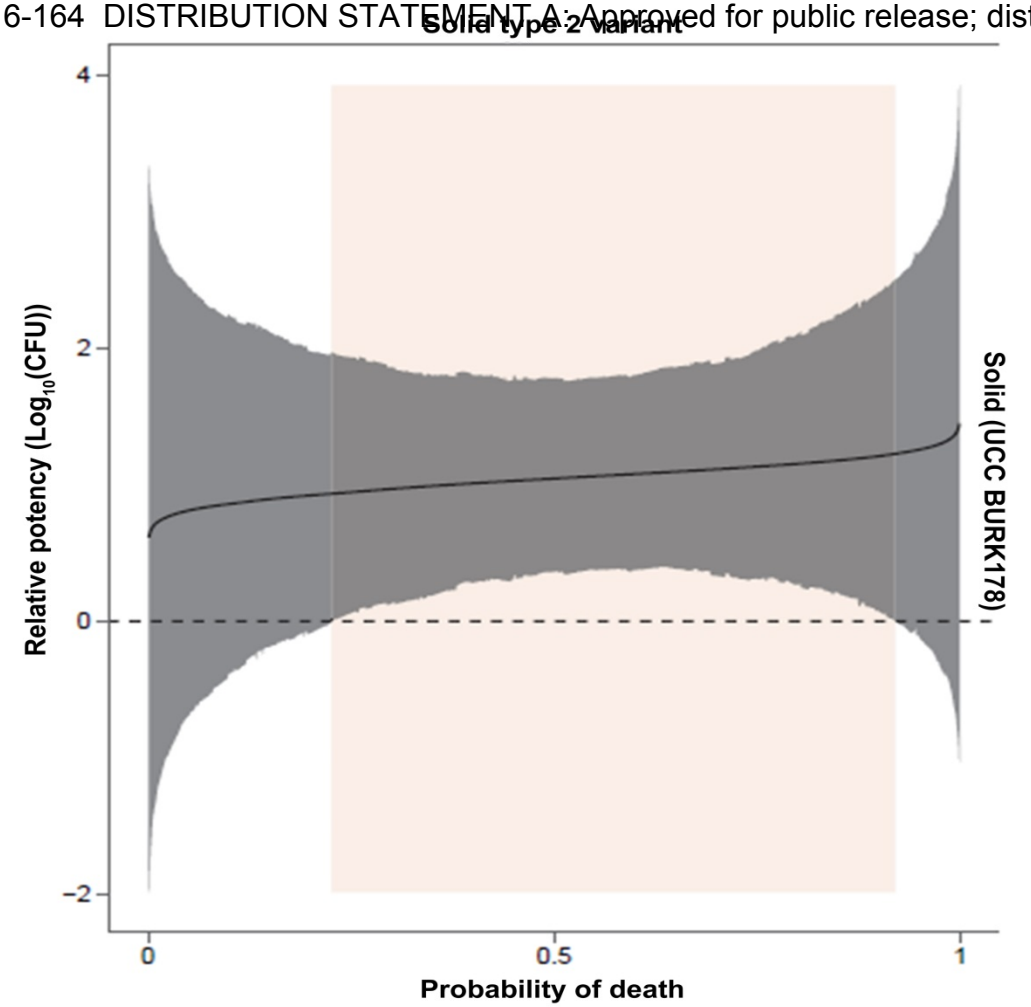

Fig S-2. The virulence of Type 1 and 2 was compared using BALB/c mice challenged by the IP route. In addition to having a ten-fold lower LD_{50} in this model, at the day 21 post-exposure time-point, type 2 exhibited greater potency at all doses associated with 23% to 92% lethal rates as determined by Bayesian analysis.

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Tables 1 – 9

Table 1. Colony morphologies in Original, Master Seed and Production stocks of MSHR5848: SUMMARY

	Variants[a]						
	Type 1		Type 2		Mucoid[b]		TOTAL
Stock	No. CFU	%	No. CFU	%	No. CFU	%	No. CFU
Original	3	**0.35**	843	**99.6**	0	0	846
Master Seed[c]	41	**4.7**	831	**95.1**	2	0.23	874
Production Stock[c]	326	**46.9**	352	**50.6**	17	2.4	695

[a]Data shown are the total no. colonies on triplicate plates for each of two dilutions.
[b]Unstable random variants of type 1 (mucoid with irregular or unformed, runny edge)
[c]Stocks were made using solid media.

Table 2 - Summary of phenotypic differences of *B. pseudomallei* MSHR5848 (BURK178) variants

Phenotype	Difference	Type 1[a]	Type 2[b]
Morphologic:			
Microscopic -			
Gram stain	Yes	GNR	typical *Bp*
PI nucleic acid dye	Yes	positive	negative

Solid media -			
Colony morphology	Yes	raised,yellowish, shiny	flat, grey, dry
BCSA Sugar utilization	Yes	alkaline	acid
Biochemical:			
Species identification			
BiOLOG GENIII	Yes	*Bp* *B. thailandensis*	4 *Burkholderia spp* *Bp*
Vitek2™	No[c]	*Bp*	*Bp*
SherlockMIDI	No[c]	*Bp*	*Bp*
Antimicrobial sensitivities	Yes	variable	variable
Phenotype analyses:			
GENIII microarray	Yes	many C sources	few
PM metabolic activity[f]	Yes	more active overall (59 significant)	less active overall (47 significant)
Molecular			
DNA sequences	Yes	WGS differences[g]	30[chrom 1], 14[chrom 2]
MLST[h]	No	-	-
Riboprinting	No	-	-
Infection:			
Macrophage phagocytosis:			
cytotoxicity	Yes	greater	reduced
replication	Yes	more	less
Mouse virulence (IP)	Yes	less virulent	more virulent[i]
Other:			
LPS banding pattern	No	Type A subtype3	Same
Persister phenotype[j]	No	positive	Same

[a]The type 1 morphotype from the solid medium production stock of strain MSHR5848.

[b]Phenotypes of the Type 2 derivative of MSHR5848 solid medium production stock are similar to those of the MSHR5848 liquid medium production stock.

[c]The Vitek2™ and SherlockMIDI identification systems reported no differences; all variants were *B. pseudomallei* (A. Shea)

[d]Inter-experimental variation in sensitivities of the type 1 and type 2 strains to selected chemicals and antibiotics in manual microtiter assays.

[e]Based on GENIII plates (94-phenotypes) incubated and analyzed on the Omnilog™

[f]Metabolic activity of variants for 1920 substrates or inhibitors (20 PM plates) meaured during incubation in the Omnilog™.

[g]Sequences from WGS libraries of variant 1 and 2 colonies were obtained using the Illumina MiSeq platform. For chromosome 1, 30 sequence variations were identified (14 in noncoding, 16 in coding regions). For chromosome 2, 14 sequence differences (6 in noncoding, 8 in coding regions) observed. One of two high confidence difference calls was in chromosome 1 gene for inorganic pyrophosphatase (frameshift and early protein truncation in type 1).

[h]The multilocus sequence type (MLST) was no. 553 for both variants; their Ribotyping Riboprinter™ rRNA patterns were conserved.

[i]LD_{50}s (days 21 and 60) of MSHR5848 Solid Type 2 was ten-fold lower than MSHR5848/Solid.

[j]This phenotype is based on an in vitro model for development of tolerance to high levels of ciprofloxacin. Both strains exhibited persister tolerance.

Table 3. Growth conditions used to produce colony morphotype switching in MSHR5848 variant colony stocks[a]

Condition:				Incubation:			Colony reversion[b]	
No.	Medium	Addition	Temp	Atmosphere	Shaking	Time and sampling	type 1[c]	type 2[c]
1	Distilled water	-	37C	aerobic	no	24h	0/11	0/11
2	TSB, pH 7.4	-	37C	aerobic	no	24h	"	2/2
3	TSB, pH 4.0	-	37C	aerobic	no	24h	"	2/2
4	TSB, pH 8.5	-	37C	aerobic	no	24h	"	2/2
5	TSB, pH 7.4	-	42C	aerobic	no	24h	"	2/2
6	TSB, pH 7.4	350mM NaCl	37C	aerobic	no	24h	"	1/1
7	TSB, pH 7.4	50mM $NaN0_2$	37C	aerobic	no	24h	"	1/1
8	TSB, pH 7.4	2 mM H_2O_2	37C	aerobic	no	24h	"	0/11
9	TSB, pH 7.4	-	37C	aerobic	no	7 days	"	4/4
10	TSB, pH 7.4	-	37C	hypoxic[d]	no	24h	"	0/11
11	LB	-	37C	aerobic	yes	serial plating, 2-72h[e]	"	0/11
12	SBAP	-	37C	aerobic	no	3d	"	0/11
13	LB	ciprofloxicin	37C	aerobic	no	stationary phase culture[f]	"	0/11
14	TSB, pH 7.4	-	37C	aerobic	yes	24h[g]	6/6[h]	nd

[a]Single colony stocks of each variant were prepared, 21 of type 1 and 17 of type 2. Growth conditions tested for reversion (#1 - 14) are described in the Materials and Methods.

[b]Number of single colony stocks with revertants to the other type for the total number stocks tested. The mean frequency of reversion to the other type for each condition ranged from 0.8 to 21% (range of of 1 - 75% in individual tests).

[c]A total of 17/21 type 1 colony stocks were tested in conditions #1 – 12 and 14; and 13/17 colony type 2 stocks were tested in conditions #1 – 13.

[d]Incubated in GasPak jar with an anaerobic gas-generating system.

[e]As described by C. Austin et al., 2015

[f]Serial plating (0h-30h) of stationary phase culture, as described by A. Butt et al., Biochem. J., 2014

[g]Performed with freshly isolated type 1 revertant from type 2 colony stock 1, after the latter had been incubated 7 days at 37C (condition #9).

[h]The six type 1 revertants produced type 2 variants in condition #14.

Table 4. Species identification of MSHR5848 variant using GENIII plates

		Statistical probability (3 tests):[b]		
MSHR5848 strain	Species identification[a]	1	2	3
Solid type 1	*B. thailandensis B*	0.932	0.938	0.954
Solid type2	*B. pseudomallei*	0.741	0.711	0.719
Liquid	*B. pseudomallei*	0.710	0.833	0.711

[a]Based on the Omnilog® species identification software as determined in three independent experiments with GENIII plates incubated for 48h. For each strain, four species with the highest probability are provided by the program; the first choice only is shown. The next most probable identification for Solid type 2 and Liquid was *B. thailandensis*, with probabilities from 0.164 to 0.240. Incubation for 36 h yielded different results, as described in the text.

[b]The values are the probability (decimal) that the test sample is the species identified, assuming the test strain is included in the database.

TABLE 5. Responses of MSHR5848 variants to selected MIS fatty acid Peaks

	Peaks[a]								
Variant	15:1 w6c	16:1 w7c/16:1 w6c	17:1 w7c	17:0 cyclo	18:1 w7c	18:1 w9c	17:0 iso 3OH	19:0 cyclo w8c	19:1 w6w/w7c/19cy
Solid type 1	0.16	8.72	0.16	6.84	31.16	-	-	5.28	-
Solid type 2	-	2.39	-	15.43	8.19	0.39	0.24	19.06	0.19
Liquid (type 2)	-	2.06	-	16.55	6.89	0.32	0.26	21.83	0.18

[a]Values shown are the percentage of the peak in relation to the whole profile

peak present in type 1 but not type 2

peak present in type 2 but not type 1

Table 6. Metabolic profiling of MSHR5848 variants using the 94 GENIII phenotypes[a]

Phenotype	Mean threshold values[b] Type 1 (solid)	Type 2 (solid)	Type 2 (Liquid)
N-Acetyl-D-Glucosamine	93.0	33.3	35.3
N-Acetyl-D-Galactosamine	97.0	30.0	29.3
D-mannose	93.0	71.7	66.7
D-sorbitol	90.7	57.0	56.3
D-mannitol	93.0	47.7	42.7
myo-inositol	96.0	59.3	63.7
Glycerol	95.0	16.0	16.3
Glycyl-L-Proline	47.7	8.3	6.3
L-Arginine	96.0	4.7	4.3
L-Aspartic Acid	92.7	17.0	17.0
L-Pyroglutamic Acid	96.3	8.7	8.7
L-Serine	94.0	41.7	44.3
D-Gluconic Acid	95.3	54.3	51.3
Citric Acid[c]	91.7	21.7	20.3
α-Keto-Butyric Acid	94.0	85.7	81.3

[a]A total of 32 substrates (of 71 carbon sources and 23 antimicrobial chemicals) differed between the 3 strains in at least 1 of 3 tests done. Included in the table are the **15** substrates which consistently differed between strains in all 3 tests.

[b]The MSHR5848 variants were incubated in GENIII plates on the Omnilog®, and the data were analyzed by the Retrospect software. The respiratory activity, reported as arbitrary OmniLog units (OL), was normalized on a 0-100 scale (Threshold values), for differences in metabolism by the bacteria of the chemicals. Positive/negative cut-off values, are in the table below.

[c]The type 2 variants' values were within 2 units of the cut-off (20) for the Threshold negative group

Threshold groups (cut-off values):	
negative	X≤20
borderline	20<X<80
positive	X≥80

Table 7A. Differences in responses of MSHR5848 Variants to PM Substrates: Type 1

PM Plate	No. wells	Substrate	Class or Mode of Action
1	6	L-Threonine	C-Source
		Citric Acid	C-Source
		L-Asparagine	C-Source
		D-Glucose-1-Phosphate	C-Source
		D-Galactonic Acid-g-Lactone	C-Source
		N-Acetyl-D-Glucosamine	C-Source
2	3	L-Pyroglutamic Acid	C-Source
		Putrescine	C-Source
		D,L Carnitine	C-Source
10	2	pH 4.5	pH, growth at 4.5
		pH 4.5 + L-Methionine	pH, decarboxylase
13	1	Thallium (I) acetate	toxic cation
14	1	Cadmium Chloride	transport, toxic cation
15	1	Guanidine hydrochloride	membrane, chaotropic agent
16	3	Chloroxylenol	Fungicide, disinfectant
		Dichlofluanid	fungicide, phenylsulphamide
		Potassium Tellurite	transport, toxic anion
17	1	Aminotriazole	histidine biosynthesis, catalase
18	3	Ketoprofen	anti-capsule
		2- Phenylphenol	DNA intercalator

		Tinidazole	Mutagen, nitroimidazole
19	2	Umbelliferone	DNA intercalator
		Thioglycerol	reducing agent, thiol, adenosyl methionine antagonist
20	2	Captan	fungicide, carbamate, multisite
		8-Hydroxyquinoline	chelator, lipophilic

*The top 25 chemicals for which Type 1 exhibits greater growth or resistance than type 2.

Table 7B. Differences in responses of MSHR5848 Variants to PM Substrates: Type 2*

PM Plate	No. wells	Substrate	Class or Mode of Action
4	15	L-Methionine	S-Source
		N-acetyl-D,L Methionine	S-Source
		L-Cysteine	S-Source
		D-Methionine	S-Source
		L-Methionine Sulfoxide	S-Source
		L-Cysteinyl-Glycine	S-Source
		Glycyl-L-Methionine	S-Source
		Glutathione	S-Source
		Cystathionine	S-Source
		Lanthionine	S-Source
		D-Cysteine	S-Source
		Thiophosphate	S-Source
		Dithiophosphate	S-Source
		L-Djenkolic Acid	S-Source
		D,L-Ethionine	S-Source
7	5	tryptophan-Asp	N-source, dipeptide
		tryptophan-tyrosine	N-source, dipeptide
		tryptophan-phenylalanine	N-source, dipeptide
		phenylalanine-tryptophan	N-source, dipeptide

		phenylalanine-Serine	N-source, dipeptide
15	1	Domiphen bromide	membrane, detergent, cationic, fungicide
17	1	Phenylarsine Oxide	tyrosine phosphatase
19	3	Iodonitro Tetrazolium Violet	respiration
		Coumarin	DNA intercalator
		Harmane	imidazoline binding sites, agonist

*The top 25 chemicals for which Type 2 exhibits greater growth or resistance than type 1.

Table 8-A. Variations identified between the type 1 and type 2 consensus sequences for chromosome 1*

type 1 Chr 1	type 1	type 2	coding/noncoding	gene	amino acid change
295173	T	G	noncoding		
295182	T	G	noncoding		
295184	C	G	noncoding		
295187	C	G	noncoding		
295191	T	G	noncoding		
295193	C	G	noncoding		
295196	C	G	noncoding		
295200	T	G	noncoding		
295202	C	G	noncoding		
295205	C	G	noncoding		
295209	T	G	noncoding		
295211	C	G	noncoding		
295214	C	G	noncoding		
902853	A	G	coding	inorganic pyrophosphatase	L (T1) to P (T(2)
902858	A	-	coding	inorganic pyrophosphatase	frameshift/early stop
902861	G	-	coding	inorganic pyrophosphatase	frameshift/early stop
902864	A	G	coding	inorganic pyrophosphatase	S (T1) to A (T2)
1048772	A	G	coding	DP65_913;hypothetical protein	Y (T1) to C (T2)
1048870	C	G	coding	DP65_913;hypothetical protein	R (T1) to G (T2)
1048877	G	A	coding	DP65_913;hypothetical protein	C (T1) to Y (T2)
1048882	A	G	coding	DP65_913;hypothetical protein	R (T1) to G (T2)
1048885	C	G	coding	DP65_913;hypothetical protein	R (T1) to G (T2)
1048892	G	A	coding	DP65_913;hypothetical protein	C (T1) to Y (T2)
1048900	C	G	coding	DP65_913;hypothetical protein	R (T1) to G (T2)
1683850	A	G	coding	membrane protein; WP_024429882.1	V (T1) to A (T2)
1934795	T	-	coding	ABC transporter permease	frameshift
1934800	T	C	coding	ABC transporter permease	synonymous
2341229	C	G	noncoding		
2599306	A	G	coding	bifunctional purine biosynthesis protein PurH	V (T1) to A (T2)
3324042^3324043	-	C	coding	2-dehydro-3-deoxyphosphooctonate aldolase	frameshift

* The high confidence coding call is highlighted. The remaining variants require further confirmation and Sanger sequencing.

Table 8B. Variations identified between the type 1 and type 2 consensus sequences for chromosome 1*

type 1 Chr 2	type 1	type 2	coding/noncoding	gene	amino acid change
328674	G	A	coding	MexH family multidrug efflux RND transporter periplasmic adaptor subunit	A (T1) to V (T2)
401708	T	-	noncoding	hypothetical protein; WP_038760828.1	frameshift

401709	A	-	noncoding	hypothetical protein; WP_038760828.1	frameshift
401710	T	-	coding	hypothetical protein; WP_038760828.1	frameshift
599753	C	G	noncoding		
599756	C	G	noncoding		
599769	T	G	noncoding		
1214371	A	C	noncoding		
1661853	T	C	coding	hypothetical protein; WP_004523585.1	synonomous
1815004	C	-	coding	4-hydroxybenzoate transporter	frameshift
2356938	T	-	coding	pyridoxamine 5'-phosphate oxidase	frameshift; GG (T1) to SR (T2); otherwise no change overall
2356943	T	C	coding	pyridoxamine 5'-phosphate oxidase	
2356941^2356942	-	G	coding	pyridoxamine 5'-phosphate oxidase	
1711179^1711180	-	C	coding	ATPase AAA	frameshift

*The high confidence coding calls are highlighted. The remaining variants require further confirmation and Sanger sequencing.

Table 9. Comparison of relative virulence for mice of strains derived from *B. pseudomallei* MSHR5848[a]

	Day 21			Day 60		
		95% Credible Interval			95% Credible Interval	
Strain	Dose, CFU	Lower	Upper	Dose	Lower	Upper
Type 1	5.18×10^3	1.67×10^3	1.7×10^4	1.12×10^2	3.58	8.98×10^2
Type 2	4.69×10^{2b}	1.38×10^2	1.42×10^3	15.1[b]	0.01	1.51×10^2

[a]BALB/c mice were challenged by the IP route with the type 1 or type 2 variant of *B. pseudomallei* MSHR5848, and the mice were monitored for morbidity and mortality for 60 days. The day 21 and 60 survival data were evaluated statistically to determine LD_{50}s and compare virulence potencies by using Bayesian probit analysis as described in the methods.

[b]Both the day 21 and day 60 LD50s of the type 2 were approximately ten-fold less than that of the type 1 morphotype; and the day 21 LD_{50} values were significantly different with a probability $\geq$95% by Bayesian probit analysis.

SUPPLEMENTARY TABLES:

Table S-1. Variants derived from BURK178 master seed and production stock morphotypes - Summary

		Variants produced from colony types - No. CFU[a]				
		Types 1 and 2		Unstable variants[c]		
Stock[b] and Colony type	Dilution	Yellow (type 1) (%)	Rough white (type 2) (%)	Mucoid (%)[b]	Flat smooth gray (%)[b]	Total No.
MSS rough white (**type 2**)	10-7	0	34	0	0	34
	10-6	0	251	0	0	251
		0 (0)	285 (100)	0 (0)	0 (0)	285
MSS Yellow (**type 1**)	10-7	33	0	5	0	38
	10-6	413	0	21	0	434
	Total:	446 (92.3)	0 (0)	26 (2.6)	0 (0)	472
MSS Mucoid	10-6	71	0	31	2	104
	10-5	629	0	108	42	779
	Total:	700 (79.3)	0 (0)	139 (15.7)	44 (5.0)	883
PS rough white (**type 2**)	10-7	0	38	0	0	38
	10-6	0	266	0	0	266
	Total:	0 (0)	304 (100)	0 (0)	0 (0)	0
PS Yellow (**type 1**)	10-7	116	0	7		123
	10-6	1056	0	50		1112
	Total:	1172 (94.9)	0	57 (1.1)	6 (0.5)	1235
PS Mucoid	10-7	102	0	9	0	111
	10-6	833	0	82	14	929
	Total:	935 (90.8)	0	90 (8.7)	14 (1.4)	1030

[a]The stocks were cultured on SBAP for 3 days at 37C and an isolated colony of the indicated type was suspended in PBS. The suspension was adjusted to OD620 = 1.0, serially diluted and the dilutions spread on each of three plates. The values shown are the total no. colonies on the triplicate plates of each dilution; the data are representative.

[b]MSS - Master seed stock. PS - Production stock

[c]Unstable variants produced by yellow type 1 colonies: a smooth flat grey variant, and large raised mucoid variants with irregular edge or unformed runny edge

Table S-2. PM substrates with greatest differences in MSHR5848 variant responses

Large Excel file, provided upon request.